DEVELOPMENT OF AN ON-FARM TEST METHOD TO DETECT BETA-LACTAM ANTIBIOTIC RESIDUES IN RAW MILK

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DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and has not been presented in this or any other University for any degree.

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DEDICATION

I dedicate this dissertation to God Almighty, my cherished family, and the multitude of steadfast friends who have been the bedrock of my academic journey. Your unwavering support and guidance have been my constant companions, guiding me through challenges and pushing me to exceed my own expectations.

To my beloved daughters, Lucy and Peninah, you hold a special place in my heart and have been pillars of strength throughout this arduous journey. Your unconditional love and unshakable belief in my abilities have been my greatest source of inspiration. My gratitude extends to my parents, Mr. and Mrs. Paul Ndungu, whose sacrifices and boundless encouragement have shaped my scholarly pursuits and personal growth. Your unwavering commitment has been my driving force, and I am humbled by the values of determination and diligence you instilled in me.

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ABSTRACT

There exist different methods of determining antibiotic residues in milk. However, the ideal testing methods such as HPLC and rapid kits have been found to be expensive, takes long to give results and needs definite expertise posing a challenge to their utility among farmers and the cooperatives societies in the Kenyan milk industry. The Hardy Diagnostics Beta-Lactamase Test (HDBT) is recommended for testing beta-lactamase production by *Neisseria gonorrhoeae, Haemophilus*, and *Staphylococcus species*. HDBT is an acidometric method that utilizes a reagent consisting of penicillin, sodium chloride, trisodium citric acid, trisodium phosphate, and phenol red dissolved in distilled water. The objective of this study was to modify the ingredients of the HDBT reagent in order to develop a test method for detecting beta-lactam antibiotic residues in raw milk. The first part of the study involved modifying the composition of the ingredients to achieve the best colour differentiation between positive and negative raw milk samples, determining the appropriate mixing ratios for the raw milk and reagent, and investigating the impact of different breeds on the test results. The second part of the study focused on determining the shelf life of the test reagent for its applicability in the dairy sector. In the third part of the study, the sensitivity and specificity of four rapid antibiotic test methods were compared to the developed test method. Pooled raw milk samples were collected from 3 Friesian and 3 Ayrshire lactating cows that had previously been treated with beta-lactam antibiotics after being diagnosed to have subclinical mastitis. Trained panelists conducted evaluations to assess the colour differences between positive and negative raw milk samples in all experiments. The results demonstrated that the gradual addition of trisodium phosphate and phenol red in the reagent resulted in a significant difference ($P \leq 0.05$) between positive and negative raw milk samples in terms of colour assessment. Additionally, mixing equal portions of milk and reagent showed a significant difference ($P \leq 0.05$) compared to other proportions, and there was no significant difference ($P \leq 0.05$) in the test method results between Friesians and Ayrshires raw milk samples. The second part of the study revealed that the reagent remained effective for 5 hours, but when used in powder form and packaged under semi-vacuum conditions, it had a shelf life of over 4 months. There was no significant difference ($P \leq 0.05$) between the results obtained using the powder and reagent. In the third part of the study, the developed test method exhibited a sensitivity of 66.7% and a specificity of 100%, while the other test methods achieved a sensitivity and specificity of 100% each. The Kappa coefficient, according to the Landis-Koch scale, indicated a moderate agreement of 0.5882 between the developed test method and the other test methods. A fuchsia purple colour indicated a beta-lactam negative sample, while peach or pink colour indicated a positive sample. This test method can be employed along raw milk collection routes to accept, set aside, or reject raw milk suspected of containing antibiotic residues. Further research on the developed test method can be carried out to investigate the possibility of obtaining quantitative results.

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LIST OF ABBREVIATIONS AND ACRONYMS

AI	Artificial Insemination
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BL	Beta Lactam
BLs	Beta-lactamases
BsDA	Bacillus Stearothermophilus Diffusion Assay
BSE	Bovine Spongiform Encephalopathy
BTS	Beta Tetracycline Sulfa
CMTK	California Mastitis Test Kit
CRD	Completely Randomized Design
CSF	Cerebrospinal Fluid
CV	Coefficient of Variation
DoF	Degree of Freedom
EC	Enzyme Commission
EC	Enzyme Commission
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
EUREC	Egerton University Ethics Review Committee
FAO	Food and Agricultural Organization of the United Nations
FMD	Foot and Mouth Disease
FSA	Food Standards Agency
GDP	Gross Domestic Product
GLM	General Linear Model
HDBT	Hardy Diagnostic Beta-lactamase Test
HPLC	High Performance Liquid Chromatography
HPTLC	High-Performance Thin Layer Chromatography
ICT	Information and Communications Technology
KDB	Kenya Dairy Board
KES	Kenya Shillings
KIPI	Kenya Industrial Property Institute
LC-MS	Liquid Chromatography Mass Spectrometry
LODs	Limits of Detection

LPS	Lacto Peroxidase System
LSD	Least Significant Difference
MRLs	Maximum Residue Limits
MRSA	Methicillin Resistant *Staphylococcus Aureaus*
MSSA	Methicillin- Susceptible *Staphylococcus Aureaus*
NACOSTI	National Commission for Science, Technology and Innovation
NAG	N-acetylglucosamine
NAM	N-acetylmuramic
NAR	Ndungu Antibiotic Residues
ND	New Castle Disease
NDTI	Naivasha Dairy Training Institute
NPV	Negative Predictive Value
PBPs	Penicillin- Binding Proteins
PPV	Positive Predictive Value
RCBD	Randomized Complete Block Design
RVF	Rift Valley Fever
SAS	Statistical Analysis System
SCC	Somatic Cell Count
SDCT	Structured Data-Capturing Technique
SDCT	Selective Dry Cow Therapy
SDGs	Sustainable Development Goals
SOV	Source of variation;
SPNT	Sulphadiazine Penicillin No Tablet
TLC	Thin Layer Chromatography
TSP	Trisodium phosphate
UHT	Ultra High Treatment
USA	United State of America
USAID	United States Agency for International Development
USD	United States dollar
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Antibiotic residues refer to small amounts of drugs or their active metabolites that remains in tissues or products such as meat, milk, and eggs from treated animals (Asredie & Engdaw, 2015; Sachi *et al.,* 2019). After lactation treatment, the presence of antibiotic residues gradually decreases over time (Hassan & Orooba, 2017). The persistence of antibiotic residues in milk depends on factors such as the milk production during treatment, the type and dosage of antibiotics used, the medium in which antibiotics are formulated, and the health condition of the animal. Administering multiple antibiotics through different routes can increase the likelihood of antibiotic residues due to the extended withdrawal period. Furthermore, variations in antibiotic dosage, treatment duration, or the use of multiple antibiotics can also raise the occurrence of residues in milk (Nirala *et al.,* 2017). Other contributing factors to the persistence of residues in milk include poor treatment records and management practices, insufficient guidance on withdrawal periods, off-label use of antimicrobial drugs, easy accessibility of antibiotics, inadequate enforcement of regulations on antimicrobial use, and limited consumer awareness regarding associated risks (Khatun *et al.,* 2018).

Presence of antibiotic residues in milk not only affects its quality but also poses significant health hazards to consumers (Nyokabi *et al.,* 2021; Wu *et al.,* 2019). After consumption of products with residues, risk of allergic reactions increases (Arsène *et al.,* 2022; Gallagher, 2015), toxicity, carcinogenicity, and hindrance of absorption certain food products (Asredie & Engdaw, 2015). Additionally, there is an increase in livestock antibiotic-resistant pathogens, leading to higher costs of disease treatment. As available drugs become less effective in managing diseases, they previously controlled, fatalities may occur (WHO, 2017). The Kenya Dairy Board (2017) estimated the costs associated with antibiotic resistance incidents to be KES 4.3 billion per year. This poses a significant economic loss, particularly for small-scale farmers whose livelihoods heavily depend on livestock production. The widespread use of antibiotics has created potential residue problems in dairy products, rendering them unfit for human consumption (FAO/WHO, 2018). In addition, presence of residues in raw milk has a significant impact on the manufacturing process of various dairy products (Asredie & Engdaw, 2015; Nyokabi *et al.,* 2021). These residues can lead to the development of undesirable flavors that persist even after processing (Adetunji,

2011) and inhibit the growth of dairy starter cultures, resulting in significant losses (Gajda *et al.*, 2018).

World-wide findings indicate that antibiotics, particularly beta-lactams, penicillins, and cephalosporins, were the most commonly reported antibiotics, accounting for 36.54% of the cases (Sachi *et al.*, 2019). Several studies conducted in Kenya indicate that many animal products in the market have unacceptable levels of drug residues (Ahlberg *et al.*, 2016; Ndungu *et al.*, 2016; Wanjala *et al.*, 2018). Ahlberg *et al.* (2016) reported the detection of beta-lactam, sulfonamides, and tetracycline at rates of 5%, 2.5%, and 0.6%, respectively, in Nandi and Makueni Counties. Orwa *et al.* (2017) reported that antibiotic residues contributed to milk losses of 23% in rural settlements and 83.5% in peri-urban settlements. Presence of residues in milk has been associated with the treatment of mastitis after the detection of high somatic cell counts (Gomes & Henriques, 2016; Gussmann *et al.*, 2017). Ali *et al.* (2017) reported similar findings in Kenya in the management of mastitis indicating high prevalence.

The bacteria responsible for causing mastitis include *Staphylococcus aureus, Streptococcus agalacteae,* and *Escherichia coli* (Gomes & Henriques, 2016; Ondiek *et al.*, 2013). These bacteria produce beta-lactamase enzymes as a survival strategy following treatment with beta-lactam antibiotics (Konaklieva, 2014; Larcher *et al.*, 2022). These enzymes can be detected in raw milk and urine after treatment, and they are referred to as endogenous beta-lactamases. The presence of these enzymes in raw milk indicates the presence of beta-lactam antibiotic residues, which is a public health concern (Wang *et al.*, 2013; Zhang *et al.*, 2020). According to Gaare *et al.* (2012), a "novel beta-lactamase induction-based principle can be applied for the specific detection of beta-lactam antibiotic residues in dairy foods". The Maximum Residue Limits (MRLs) for beta-lactam antibiotics are set at 4 µg/L, which is significantly lower compared to 100 µg/L for tetracycline and sulfonamides, making them more detectable at higher levels (FAO/WHO, 2018). Differences exist in the methods used for testing antibiotic residues, including the type of test, susceptibility of test groups of antibiotics, determination procedures, and financial requirements (Gondova *et al.*, 2014). In the European dairy industry, losses associated with milk contaminated with antibiotics amounted to over 200 million euros annually (Sachi *et al.*, 2019; Technische Universitaet Muenchen, 2010). In developing countries like Kenya, the risks associated with milk residues are higher due to inadequate detection methods and insufficient enforcement by regulatory bodies (Kebede *et al.*, 2014). Particularly in developing countries detection facilities are often limited (Sachi *et al.*, 2019) in terms of control systems applicability and therefore unsustainable. In addition, there is inadequacy of

rapid antibiotic residues testing in the Kenyan milk value chain, specifically in smallholder systems (Orwa *et al.*, 2017). It is crucial to develop highly sensitive detection tools as a quality control and preventive measure to avoid false negative results (Sachi *et al.*, 2019) and untested milk into factories or end consumers. Furthermore, there is a need for simple and cost-effective quality control tests suitable for field situations (Prajwal *et al.*, 2017).

The comprehensive evaluation of antibiotic residues typically requires advanced techniques like immunological tests, microbiological approaches, vibrational spectroscopy liquid chromatography and mass spectrometry. Although these methods provide exceptional precision and resolution, they necessitate specialized knowledge and substantial financial resources (Salois *et al.*, 2015; Tuei *et al.*, 2021). Some of these methods also have longer turnaround times, making them unsuitable for rapid milk acceptance as platform tests. Platform tests are rapid quality control tests used to determine whether milk should be accepted or rejected (Ndungu *et al.*, 2016). In the Kenyan market, there are several simple and rapid antibiotic residues kits available, including sulfa drugs, tetracycline, Ringbio beta-lactam BLQ, Rapid Test Kit, Mtusbio beta-lactam, Delvo Sulphadiazine Penicillin No Tablet (SPNT) and Delvo test Fast BL and BTS 3 in 1 Tri Test S. These kits require less technical expertise, provide quick results, but may sometimes yield conflicting results (Mullen *et al.*, 2017) thereby limiting their use in raw milk acceptance. Some of these kits also require incubation in their analysis procedure, and since electricity is not readily available in the milk collection routes, it poses a challenge. Moreover, the general cost per sample for these kits is high (approximately three hundred Kenya shillings per strip) and unsustainable, especially considering the large number of samples involved in the Kenyan milk collection systems.

The acidometric approach known as the Hardy Diagnostics Beta-Lactamase Test (HDBT) is a recommended method for assessing the presence of beta-lactamase production in Staphylococcus, Haemophilus and Neisseria gonorrhoeae species. The HDBT reagent comprises penicillin, sodium chloride, trisodium citric acid, trisodium phosphate, and a phenol red indicator. The presence of penicillin in the reagent triggers a hydrolysis reaction with the beta-lactamase enzyme produced by the bacteria. The amount of beta-lactamase produced determines the extent of hydrolysis of the beta-lactam ring, resulting in the formation of penicilloic acid. Sodium chloride promotes optimal growth of the microbes, while trisodium citric acid and trisodium phosphate together form the Mcllvaine buffer, which maintains the necessary pH conditions for the reaction. The phenol red indicator undergoes colour changes as penicillin is hydrolyzed by the beta-lactamase enzyme. During a specific incubation period, there is a decrease in pH, leading to a color transition of the

reagent from red to yellow. This change is observed through the phenol red indicator. A positive test result is indicated by the reagent turning yellow within the specified time range of 2 to 60 minutes. On the other hand, a negative reaction is indicated when there is no colour change (the reagent remains pink).

Dairy production plays a crucial role in supporting economies and livelihoods in East Africa, with Kenya being the leading milk producer in the region, producing over five billion liters of milk annually. The dairy industry plays a substantial role in the economy of Kenya, contributing around 14% to the agricultural GDP, 3.5% to the total GDP and 40% to the livestock Gross Domestic Product (GDP). Smallholder dairy farmers are responsible for about 75% of the country's total milk supply. Kenya has high milk consumption rates compared to other sub-Saharan African countries, ranging between 50 and 150 liters per capita per year (Alonso *et al.*, 2018; Bosire *et al.*, 2017). Kenya as a country has plans encompassed in the Vision 2030 and Sustainable Developments Goals (SDGs) to improve agricultural sector. Vision 2030 is Kenya's long-term development plan aimed at transforming the country into a middle-income nation with a high quality of life by 2030 in which food safety and security is given a priority. It focuses on three pillars: economic, social, and political. The economic pillar seeks inclusive growth in sectors like tourism, agriculture, manufacturing, and ICT. The social pillar emphasizes education, healthcare, housing, and social security, while promoting equity and gender equality. The political pillar aims to enhance governance, institutions, peace, security, and justice. In parallel, Kenya is committed to implementing the Sustainable Development Goals (SDGs), a global agenda for sustainable development encompassing 17 goals. Kenya has integrated the SDGs into its national policies, collaborating with stakeholders to monitor progress. Some of the stakeholders include the universities, researchers, and technical institutes, governmental and non-governmental organizations. Initiatives like the bottom-up economic transformation agenda, targeting housing, manufacturing, healthcare, and food security, contribute to achieving the SDGs. Together, Vision 2030 and the SDGs guide Kenya's development toward a sustainable and prosperous future.

The objective of this study was to modify the HDBT reagent formula, as described previously, in order to create a straightforward, fast, and stable test method for detecting beta-lactam antibiotic residues in raw milk. The performance of this modified method was then evaluated and compared to other kits currently available in the market. The study focused on utilizing the presence of the beta-lactamase enzyme in raw milk, which is produced as a result of treatment with beta-lactam antibiotic drugs. The ultimate goal was to develop a method

that could be implemented at the farm level, specifically in milk collection routes and milk reception platforms. These stages are crucial control points for detecting residues and ensuring the enhancement of milk quality control systems and consumer safety. Coming up with easy, cheaper methods to detect antibiotic residues will highly contribute to the achievement of the SDGs 2, SDGs 3 and vision 2030 in improving agricultural sector especially dairy value chain and agro-processing (Ajwang & Munyua, 2016; Alonso *et al.*, 2018; Bosire *et al.*, 2017; Macharia, 2019).

1.2 Statement of the Problem

Kenya is on the verge of achieving its Vision 2030, with food security being a key agenda. The dairy sector plays a significant role in shaping the country's progress. However, a major challenge faced by Kenya is the presence of milk adulterants, particularly antibiotics, leading to losses and rejections. These antibiotics are often used to treat animal diseases, and some farmers fail to observe the required withdrawal periods. Mastitis, a common disease in lactating cattle, accounts for 82% of cases, and beta-lactam antibiotics are widely used for bacterial infections.

Numerous rapid methods are accessible for identifying antibiotic residues in milk; however, a considerable number lack precision and offer only partially quantitative or inaccurate positive outcomes. In order to address these challenges, advanced techniques like high-performance liquid chromatography (HPLC) have been established. These methods provide dependable, sensitive, and quantitative results. However, HPLC is costly, time-consuming, and requires expertise and resources, making it difficult for farmers to use.

The applicability of rapid test kits for antibiotic residues detection faces hurdles, such as the lack of electric power and high costs per test, posing challenges to cooperatives and milk processors in Kenya. Additionally, the structure of milk collection routes in Kenya, involving numerous smallholder farmers, makes it impractical to install electric power at collection points. Investments are primarily focused on ensuring immediate milk cooling, while testing the raw milk is often overlooked by some cooperatives and processors.

Consuming food with residues poses risks of allergic reactions, toxicity, carcinogenicity, and interference with certain food products. Given the increased health risks and microbial resistance, there is a need for effective and appropriate methods to detect residues in the dairy sector.

1.3 General Objective

To contribute to food safety through development of an on-farm test method to detect beta-lactam antibiotics residues in raw milk.

1.3.1 Specific Objectives

i. To formulate ingredients for detecting beta-lactam antibiotic residues in raw milk using beta-lactamase enzyme by modifying the HDBT reagents.
ii. To determine the formulated reagent's shelf life under different storage conditions.
iii. To determine the sensitivity and specificity of the developed test method and compare with locally available testing kits.

1.3.2 Hypotheses

i. **Ho:** Formulation and modification of HDBT reagent using Beta-lactamase enzyme has no significant potential for developing a test method for detecting beta-lactam antibiotic residues in raw milk.
ii. **Ho:** There is no significance difference on the test method reagent's shelf life under different storage conditions.
iii. **Ho:** The sensitivity and specificity of the developed test method has no significance difference with the locally available kits.

1.4 Justification

The main source of antibiotic residues primarily originates from the farm level. The detection and characterization of chemical and veterinary drug residues in animal-derived food products are areas undergoing rapid development in food processing. These advancements primarily focus on ensuring food safety. Residual substances emanating from the veterinary substances can be found in milk, eggs and edible tissues intended for consumption by humans, potentially posing varying levels of toxicity to consumers. Hence, the importance of easy, fast, and sensitive tests for effective on-site use cannot be overstated.

This research presents the latest advancements in the rapid detection of chemical and veterinary drug residues, specifically beta-lactam, in animal-derived foods, including milk. This is particularly relevant due to the presence of bacteria, such as *Staphylococcus aureus*, *Streptococcus agalacteae*, and *Escherichia coli,* which produce the beta-lactamase enzyme as the primary mechanism of antibiotic resistance against beta-lactam antibiotics after treatment for mastitis. The proposed method aims to enhance the quality control of raw milk by testing residues at the farm-level collection routes and milk reception platforms.

Ensuring milk quality control is crucial because once these residues enter the food chain; there are no processing procedures in place to eliminate them or their potential side

effects on consumers. The method presented in this research is user-friendly, fast, and allows analysts or the farmers to easily identify and differentiate colours with minimal training. Consistently conducting these tests will promote discipline among farmers in adhering to withdrawal periods and ultimately ensure consumer safety. Examining antibiotic residues in milk prior to its distribution to consumers is of paramount importance in dairy production. This practice serves to minimize the potential for lingering contamination within the food supply chain. However, the selection of an appropriate antibiotic screening test is pivotal in ensuring the effectiveness and accuracy of residue detection. The innovative method proposed by this study aims to address this challenge by providing a simpler yet efficient solution for antibiotic detection, which is particularly relevant for small-scale farmers and cooperatives in Kenya who may face difficulties with the installation and operation costs of high-performance liquid chromatography (HPLC) equipment, as well as the analyte preparation process.

In the effort of the Kenyan government to fulfill the efforts for Vision 2030 and its SDGs, the suggested method is also aimed at improving the agricultural sector especially the dairy value chain by reducing the amount of milk rejected due to presence of antibiotics drug residues. Moreover, it aids in ensuring that dairy products produced or sold in the market meet the legal requirements in terms of the maximum residue levels. This will overly assure supply of quality raw materials and reduced miss productions, for products such as cheeses and yoghurts, for manufacturers in dairy industries that would otherwise be affected by the unexpected high levels of antibiotic drug residues. This novel test method does not require expensive equipment or electrical power and yields immediate results. Implementing this research will enhance quality control systems, contribute to addressing food safety concerns, ensure the quality of finished products, and reduce losses experienced by manufacturing industries.

CHAPTER TWO
LITERATURE REVIEW

2.1 Kenya's Dairy Industry and Dairy Production Systems

In Kenya, two main categories of cattle are reared for milk production and various other uses: the first is composed of indigenous zebu cattle, and the second comprises exotic breeds and their hybrids, commonly referred to as dairy cattle. The exotic and their hybrids contribute approximately 60% of production of milk nationally, while indigenous breeds contribute the remaining 40%. The main dairy zones in the country are Rift Valley and Central Provinces, which account for more than 80% of the total dairy cattle population. Zebu cattle are extensively spread throughout all provinces and agro-ecological zones in Kenya, making up approximately 70% of the overall population of the cattle. Nonetheless, the Ministry of Agriculture and Rural Development attributes the perceived lack of progress in dairy production to the absence of a comprehensive census data detailing cattle growth rate and population. This situation has led to production estimations relying on figures resembling those from the preceding two decades. (Kariuki, 2016).

The projected total milk production for 2006 was roughly estimated at 2.7 billion liters. These estimations appear doubtful when considering the recent favorable growth rate of dairy herds due to farmers diversifying away from cash crops like coffee and maize. Furthermore, milk production assessments have been based on an average yield of 1,300 liters per cow annually. However, research indicates that real yields across all production systems are considerably higher, spanning from 1,500 to 3,000 liters per cow yearly (Karanja, 2003). Thus, there exists reason to assume that the actual milk production in the nation surpasses the figures reported in official records, potentially accounting for the observation of an expanding surplus within the dairy sector (Kariuki, 2016).

Dairy farming stands as a pivotal activity within the livestock sector and serves as a vital means of sustenance for around 600,000 small-scale farmers. In addition to milk, dairy animals contribute resources such as manure and marketable products including calves and culling. They also offer intangible benefits like insurance and social status. In 1995, the assessed value of dairy production stood at KSh 23.1 billion, amounting to 14% of the overall value of agricultural production. By the year 2000, milk production surged to an estimated 2.3 billion liters, with 63% allocated for market, 30% for domestic consumption, and the remaining 7% utilized as calf feed (Kuriyan, 2002). The calculated value of this production was KSh 35.2 billion, equivalent to 25% of the total agricultural output registered in 2001

(Kariuki, 2016). Based on these findings, it is necessary to conduct population statistics to accurately determine the cattle population, actual production, and associated incomes.

Despite the significant contribution of the dairy industry to the national economy and household incomes, it faces numerous technical, economic, and institutional challenges, which have seemingly intensified recently. Despite smallholder dairy production contributing more than 56% and 70% to the complete milk production and marketed output correspondingly (Muriuki, 2011), the productivity per animal in these settings continues to remain at a low level. Factors such as irregular payments, low farm gate prices, low sales compared to total production (especially evening milk), unreliable market outlets, and limited access to veterinary and artificial insemination (A.I) services all have a negative impact on the productivity and performance of the dairy sub-sector. Nonetheless, there is significant potential to increase dairy productivity in the country, particularly among smallholder dairy farms. As an illustration, the average output per cow in smallholder farms stands as low as 1,300 liters annually, in contrast to the optimal international standards of 4,000-6,000 liters. (Kariuki, 2016; Kovalenko, 2019).

Smallholder dairy farmers dominate Kenya's dairy industry, accounting for up to 80% of the country's total milk production (Leone *et al.*, 2014; Majiwa *et al.*, 2013). This industry is the largest agricultural sub-sector, contributing 14% to the agricultural Gross Domestic Product (GDP) and 4% to the national GDP (KDB, 2014). It plays a vital role in addressing food insecurity and generates employment and income for various stakeholders involved in the milk supply chain, including farmers, transporters, traders, vendors, employees of dairy societies, milk processors, input suppliers, service providers, retailers, and distributors (Wambugu *et al.*, 2011). While the sale of raw milk remains prevalent, the demand for milk has led to innovative retail options such as pasteurized milk sales. Nonetheless, there is a growing demand for milk consumption in Kenya, with an annual growth rate of 7%. The current per capita milk and dairy products consumption stands at 110 liters per year and is expected to double by 2030 (Kibogy, 2019). Although this has encouraged milk production and marketing, it has also affected compliance with safety and quality standards at national, regional, or international markets. Despite efforts to raise awareness and build the capacity of producers and processors, conformity to these standards has been a challenge (Bebe *et al.*, 2019).

Previous research has estimated milk losses in Kenya to be 54.2 million liters per year, with most of these losses occurring in the small-scale informal dairy sector (Kaitibie *et al.*, 2010). While microbial contamination is a major cause of losses, there are also other

contaminants that are foreign and unacceptable in milk. The presence of contaminants like antimicrobial residues renders the milk unsuitable not only for processing but also for consumption. In addition to the high microbial load found in raw milk supplied by smallholder dairy farmers in Kenya (Kenya USAID, 2010; Ndungu *et al.,* 2016a), unlawful adulteration with chemical substances for instance hydrogen peroxide (H_2O_2) (Wanjala *et al.,* 2018) and the presence of drug residues (Ahlberg *et al.,* 2016; Kosgey *et al.,* 2018) reported in several studies. A study by Orwa *et al.* (2017) assessing sulphonamides and tetracyclines antibiotic residues in rural and peri-urban dairy value chains in Kenya found antibiotic residues in 28.8% (23/80) of the raw milk samples collected from the peri-urban area. Furthermore, 60% of all the positive samples analyzed contained sulphonamides, with 71% exceeding regulatory limits. Antibiotics were also found in high concentrations in rural farms (46.7%) and peri-urban bulking hubs (50%). Kosgey *et al.* (2018) reported the presence of antibiotic residues in milk sold through milk vending machines in Eldoret, Kenya. Other researchers have also detected residues in milk in the Kenyan market (Ahlberg *et al*., 2016; Brown *et al.,* 2020). According to Bebe *et al*. (2019), the presence of antibiotic residues indicates a failure to adhere to withdrawal periods. It is, therefore, necessary to increase consumer awareness regarding antibiotic residues in milk and its products (Wanjala *et al.,* 2018). Additionally, testing for residues in raw milk should be encouraged, especially at the farm level before bulking occurs.

2.1.1 Challenges Facing Milk Production and Milk Processors

One of the challenges encountered in dairy farming is the lack of sufficient storage facilities for dairy products. Kenyan farmers commonly face the issue of inadequate storage facilities, resulting in significant milk wastage. This problem is particularly pronounced during peak seasons and is further exacerbated by unpredictable weather patterns. The current heavy rains and floods worsen the situation, causing a shortage of milk in the country. To address this, it has been suggested that the government should provide portable storage facilities on a loan basis, similar to practices in developed countries. These facilities would ensure continuous milk production even during rainy periods (Tamanna, 2018).

Another problem in the dairy farming industry is the delay in payment to farmers. Milk processing plants often have disputes with farmers, especially regarding end-of-month payments. This significantly affects the morale of farmers, leading some to resort to direct milk sales to mitigate their losses. The Ministry of Agriculture has failed to intervene and advocate for the rights of dairy farmers, resulting in the emergence of unscrupulous

middlemen who exploit the farmers' vulnerability. The delayed payments and exploitative practices of milk processors create financial difficulties for farmers who also bear the cost of purchasing feeds and supplements (Ispirli *et al.*, 2017). County governments have been tasked with finding alternative solutions to assist farmers (Hamad *et al.*, 2018; Ispirli *et al.*, 2017).

Inadequate infrastructure is another quagmire the dairy industry faces during milk delivery. Recent heavy rains and floods in Kenya have further exacerbated the situation by damaging roads and impeding the transportation of milk to processing plants. Even in the absence of rain, the country's roads are generally in poor condition (Kariuki, 2016). Many farmers struggle to transport their products to distant milk processors, and the limited availability of milk cooling facilities proves it hard for a rural farmer to access different markets. Numerous dairy farmers reside in remote areas without proper road access, challenging them to connect with other channels and ensure timely delivery to processing companies. Although the government has made efforts to improve road infrastructure, regions with high milk production continue to face a lack of proper roads. County governments should prioritize the development and maintenance of roads to facilitate efficient milk delivery and reduce losses (Mahmud, 2019; Muriuki, 2011).

It is necessary to allocate more resources for the maintenance and development of roads, and county governments should take responsibility for connecting all constituents to a well-developed road system. This would save farmers time in delivering milk and prevent losses. Additionally, there is a need to establish more milk cooling facilities throughout Kenya. Currently, functional facilities are primarily located in major cities and a few towns, making them inaccessible to most farmers who urgently require such services. There is need to invest in cooperatives to ensure efficient milk production in Kenyan (Kilelu *et al.*, 2017) as well as on milk quality control.

Another challenge faced by dairy farmers is the cost of expensive feeds and supplements for dairy cows. To enhance milk production, farmers must purchase feeds and supplements, which often come at high prices. Due to delayed payments and low milk prices, farmers are compelled to bear the financial burden of financing these supplements. The government does not provide incentives for producing commercial feeds, thereby shifting the financial responsibility to the farmers. Moreover, the availability of regular fodder is a major issue, given the unpredictable weather patterns and frequent dry spells (Kilelu *et al.*, 2017). This scarcity of quality feeds leads some farmers to opt for cheaper but lower-quality alternatives, ultimately resulting in reduced milk production (Kilelu *et al.*, 2017). Inadequate

education on proper feeding regimes and the high costs of animal healthcare contribute to malnutrition deficiencies and diseases among dairy cows.

The availability of poor-quality feeds in the market is a recurring issue. Many farmers opt for these low-cost feeds, but they ultimately result in a reduction in milk production (Kilelu *et al.*, 2017). Additionally, farmers often lack proper education on optimal feeding practices to increase milk production. Consequently, some dairy cows suffer from malnutrition and diseases, and the high cost of animal healthcare leads to significant losses (Mbungu, 2014). Without access to commercial feeds, milk production declines significantly, and farmers are forced to find alternative feed sources. Some even resort to taking loans to purchase supplements. Dairy cows require a balanced mixture of nutrients and supplements in addition to regular fodder. Farmers who lack these essential nutrients experience low returns and incur losses. Replacing quality pasture with other feed sources does not yield any advantages in terms of milk production (Odero-Waitituh, 2017).

Dairy farming faces a lack of economies of scale, particularly among small-scale farmers in Kenya. Due to a lack of education and management skills, these farmers struggle to turn their milk production into successful enterprises (Odero-Waitituh, 2017). However, this challenge can be addressed through collective capacity building and collaboration among farmers in the same region. Establishing dairy institutions owned and operated by farmers can provide training and support for capacity building in the industry (Rademaker *et al.*, 2016). The Naivasha Dairy Training Institute (NDTI) serves as an example by training local farmers on effective methods to ensure profitability in their businesses. This issue is particularly prevalent in Uasin Gishu County, a significant milk-producing region. The lack of collective organization among dairy farmers hinders their ability to form cooperatives and marketing organizations. By pooling resources and building capacity, farmers can seek support from financial institutions and the county government to access ready markets. This strategy eliminates unscrupulous milk dealers and allows farmers to directly connect with high-demand markets (Rademaker *et al.*, 2016).

Insufficient participation by industry players in the sector is another challenge. This lack of influence in market and legislative policies at the county government level prevents favorable conditions for farmers (Ter-Hemen, 2015). Farmers are left to explore alternative means to make profits during challenging times in the dairy farming industry. They focus on improving quality, reducing wastage, and gaining easy access to markets that meet export standards. Government policies should provide incentives to motivate dairy farmers and address these challenges (Rademaker *et al.*, 2016; Ter-Hemen, 2015).

Ensuring milk safety and managing medicine residues is an essential responsibility for milk processors. In the European Union, legislation requires the recall and destruction of products that test positive for residue at the individual cow level. Developing countries can adopt similar practices through collaboration among stakeholders. Farmers shoulder the responsibility of safeguarding the raw milk's safety prior to its entry into the bulk storage tank. Milk processors depend on farmers' prudent antibiotic utilization to mitigate the potential for residues within the milk value chain. Nonetheless, during transportation, raw milk gets diluted with milk from other cows or farms, which is referred to as "natural dilution" by milk processors. This dilution can lead to milk testing below maximum residue levels (MRLs) at the processor level, even if it exceeded MRLs at the individual cow level. While the safety of the milk might be deemed acceptable, it's important to note that both farmers and milk processors could be violating EU MRL regulations if the levels are surpassed either at the level of individual cows or within bulk milk tanks. The process of natural dilution is a result of operations and not a deliberate attempt at dilution (Begemann *et al.*, 2020).

In order to safeguard dairy supply chains, the Food Standards Agency (FSA) has previously shown a lenient stance towards the phenomenon of milk residue dilution. Nevertheless, growing concern about the public health implications of antibiotic utilization in animals raised for food has compelled the FSA to push milk processors for enhanced management of residue levels. Kenyan milk purchasers are now grappling with this issue, which raises worries not just about safety but also about financial implications. Milk processors incur significant costs in investigating milk failures. Educating farmers to practice responsible antibiotic use is crucial in managing the risk of milk residues. The aim is to eliminate antibiotic contamination in milk, and efforts are made to help farmers test their milk before it is collected by milk factories (Begemann *et al.*, 2020; Zavala, 2022).

Milk processors have identified two key antibiotic practices by farmers that contribute to milk residues in the dairy supply chain: "dry cow therapy" and farmers' "off-label use" of antibiotics. Dry cow therapy refers to the treatment of cows with antibiotics during the dry period, a phase in the cow's lactation cycle when milk production is stopped for at least 40 days until the next calving. In the past, guidelines recommended blanket treatment of all cows with antibiotics during this period, leading to extensive prophylactic antibiotic use. An alternative approach known as Selective Dry Cow Therapy (SDCT) has emerged, where only cows with a low probability of infection are given a teat sealant to prevent pathogens from entering their bodies. This strategy significantly reduces antibiotic usage and is considered

the most important approach by some Kenyan milk processors to mitigate the risk of milk residue failures. Influencing farmers' drying off strategies is seen as the most effective way to have a significant impact on antibiotic usage (Begemann *et al.*, 2020).

Milk processors are also significantly troubled by the off-label use of antibiotics by farmers. This term pertains to the utilization of medications in ways that deviate from their authorized terms, potentially impacting the period during which milk should be withheld. Some farmers might not possess a complete grasp of the consequences associated with off-label use or its misapplication, which heightens the likelihood of unprocessed milk residues finding their way into the supply chain. Farmers could extend antibiotic treatments or administer additional doses, subsequently prolonging the officially stipulated withdrawal periods of the original treatments (referred to as the "topping up effect"). The lack of awareness and comprehension surrounding off-label use contributes to an elevated risk of residue (Begemann *et al.*, 2020; Kilelu *et al.*, 2017).

Milk processors regard milk residues as a substantial worry, shaping their stance on antibiotic management. These processors not only present compelling evidence of the political significance of antibiotic governance but also forge fresh economic ties with retailers by addressing milk residues as a financial hazard within dairy supply chains. Their antibiotic strategies hold farmers accountable and underscore their personal duty to supply safe milk. Diverse policy tools and strategies for behavioral change, including SDCT protocols, workshops, milk residue stewardship initiatives, penalties on milk pricing, and antibiotic test kits, are put into action to harmonize antibiotic practices with established protocols and minimize the risk of milk residues in the dairy supply chain. Nevertheless, the conversion of scientific understanding into practical application follows a non-linear trajectory, frequently encountering a collision between theory rooted in evidence and the realities of decision-making processes in the field. The adoption of knowledge tools is shaped by negotiations with local cultures and environments, highlighting the need to understand how knowledge is adapted to fit agricultural networks and concerns of farmers (Salembier *et al.*, 2020).

2.2 Challenges facing the Kenyan Dairy Industry Smallholder Production Systems

Kenya's dairy industry, which is largely dominated by smallholder farmers, plays a crucial role in the country's economy. The industry provides employment and income generation opportunities for various stakeholders involved in the milk supply chain (Wambugu *et al.*, 2011). Despite the significant contributions of smallholder dairy farmers, the industry faces several challenges that hinder its growth and potential. One major

challenge is the poor state of road infrastructure. According to a study, only 30% of households in the study area have access to good roads, which restricts their ability to do marketing of their farm produce and purchasing of inputs consistently across the year. During the rainy seasons, the situation worsens as many roads become impassable, preventing farmers from selling their products. The high transportation costs resulting from the poor road network also make smallholder dairy production less competitive (Karanja, 2003).

In addition to poor road infrastructure, the dairy industry suffers from poor marketing systems. Particularly during the wet season, a significant portion of the milk produced remains unmarketed due to the inadequate road network and long distances to markets. The perishable nature of milk, combined with the lack of milk cooling equipment for farmers, leads to increased post-harvest loss. Roughly 35% of the complete milk production is channeled through the formal sector, which is deemed to offer greater reliability in terms of milk prices and compensation. The restricted processing capacity within the formal sector compels farmers to sell excess milk through the informal sector at reduced prices. Inadequate organization of marketing systems, processing and milk collection adds another layer of hindrance to the industry's ability to efficiently access urban markets (Mbungu, 2014).

Smallholder dairy farmers are confronted with the challenge of elevated expenses and a lack of access to necessary resources and support services. Insufficient road infrastructure and lengthy distances to markets contribute to heightened costs for transportation and distribution. As a result, the prices of crucial inputs like animal drugs, supplements, vaccines, herbicides, pesticides and fertilizers are driven up. Additionally, vital resources such as herbicides, fertilizers, pesticides, vaccines, animal drugs, supplements, as well as services like artificial insemination, animal health, electricity provision, extension and training, and credit, are either costly or inaccessible within the studied region. Furthermore, the absence of electricity in rural areas inhibits investments in cold storage facilities and milk processing, thereby further impacting the sector's competitive edge. Difficulties in accessing credit from formal banking institutions, due to factors such as high costs and limited collateral options, add to the challenges faced by smallholder dairy farmers (Karanja, 2003; Mbungu, 2014).

The inadequate use of appropriate technologies in dairy farming exacerbates the industry's struggles. The high cost and inaccessibility of artificial insemination services lead to a significant reliance on natural breeding methods, resulting in genetically inferior animals and poor performance of offspring. Insufficient improved fodder production and conservation practices force farmers to depend on inadequate and poor-quality natural pastures, leading to feed shortages and overstocked farms. The limited access to extension services, coupled with

high levels of illiteracy among household heads, results in poor knowledge and skills in animal husbandry, negatively impacting dairy stock performance. The lack of appropriate technologies and the high production costs associated with dairy farming further contribute to the sector's challenges (Mbugu, 2014; Ter-Hemen, 2015).

A prevailing concern is the restricted enhancement of value in milk and dairy items. A significant portion of milk within the studied region is marketed in its fresh state through informal means, resulting in notable milk wastage due to spoilage and leakage across the value chain. Substandard hygiene practices throughout the production and marketing processes present health hazards to consumers. Inadequate regulatory measures and the incapacity to comply with global standards for food safety and quality hinder the sector's participation in both regional and global markets. This circumstance contributes to subdued milk prices and suboptimal dairy production (Ter-Hemen, 2015).

Furthermore, agricultural activities have encroached upon forests and water catchments, leading to environmental degradation. The influx of farmers into the region has led to the enlargement of settlements by humans, deforestation, and the utilization of land for both animal production and crop cultivation. This has had detrimental effects on the ecosystem, including poor soil fertility and reduced fodder yields. The lack of manure collection and use under common extensive grazing systems further exacerbates the issue. If left unchecked, these practices will lead to water pollution, environmental problems, and the loss of fauna and flora in the region (Mbungu, 2014).

The traceability of antibiotic residues in Kenya's agricultural sector faces significant challenges, particularly due to the involvement of a large number of smallholder farmers. Lack of awareness and knowledge; a study by Ombui *et al.* (2016) emphasized the limited awareness and understanding among smallholder farmers in Kenya regarding the risks associated with antibiotic residues in food products. Farmers often lack knowledge about appropriate withdrawal periods and fail to adhere to recommended dosages.

Moreover, inadequate veterinary services also castigate the problem. The limited access to veterinary services in rural areas of Kenya contributes to inappropriate antibiotic use. Smallholder farmers often lack guidance and supervision on proper antibiotic administration, exacerbating the issue (Mutua *et al.,* 2019). Weak regulatory framework is another problem associated with traceability of antibiotic residues. Kenya's regulatory framework for monitoring and controlling antibiotic residues is inadequate. Stricter regulations, improved enforcement mechanisms, and comprehensive surveillance systems are required to ensure compliance and address the issue effectively (Ngigi *et al*., 2020).

Limited laboratory capacity also plays a crucial role in the traceability process. The availability of well-equipped laboratories for antibiotic residue testing is insufficient, particularly in remote areas. This lack of adequate infrastructure hampers timely and accurate detection of residues related to antibiotic residues in livestock products (Kosgey *et al.*, 2020). Informal sector and market dynamics; the presence of an informal market in Kenya poses challenges to traceability efforts. Smallholder farmers often sell their products directly to consumers or local markets, bypassing formal channels. As a result, tracking and tracing antibiotic residues becomes challenging (Mitema *et al.*, 2020).

Overly, the dairy industry in Kenya faces multiple challenges that hinder its growth and development. These challenges include poor road infrastructure, inadequate marketing systems, high costs and unavailability of inputs and support services, limited use of appropriate technologies, and insufficient value addition of milk and dairy products. Addressing these challenges is crucial for enhancing the competitiveness and sustainability of the smallholder dairy sector in Kenya (Ter-Hemen, 2015) together with having methodologies and technologies to assist in curbing overuse or detection of antibiotics and pesticides residues. The challenges in traceability of antibiotic residues in Kenya's smallholder farming sector call for improved awareness among farmers, stronger regulatory frameworks, enhanced veterinary services, better laboratory capacity, and targeted interventions. Resolving these challenges is of paramount importance to guarantee the safety and quality of food products in Kenya.

2.2.1 Urban Milk Markets

The informal milk market in Kenya is a significant channel for the marketing of milk, with over 80% of milk from smallholder farmers and a large portion from large and medium farms being sold through this channel. The informal market primarily caters to urban centers with substantial demand, offering convenient delivery and cost advantages due to reduced processing and handling expenses. This sector is significantly influenced by small-scale milk traders, commonly referred to as hawkers, alongside farmers from rural and peri-urban regions who vend milk from their own farms. Additionally, milk bars and cottage industries function as milk vendors (Bebe *et al*., 2018). A shared trait among these informal participants is their sale of unpasteurized milk, frequently without packaging. This has raised concerns regarding potential public health hazards tied to the informal milk market. Nonetheless, it's essential to weigh the balance between ensuring quality and limiting legitimate business prospects within the Kenyan dairy sector. It's pertinent to recognize that a majority of

households in Kenya do boiling of milk prior to consumption, significantly diminishing pathogen risks (Bebe *et al.*, 2018; Mahmud, 2019).

Beyond the concerns regarding public health, there exist additional market risks linked to practices such as adulteration, utilization of non-food plastic containers, and unsanitary milk handling. These factors elevate the potential for communicable diseases. Moreover, a substantial number of informal milk traders experience notable losses due to subpar milk quality. For example, during the study, 70% of the visited milk bars reported an average monthly loss of 40 liters of milk, despite using various quality assessment methods. Some commonly used technologies to assess milk quality in these milk bars include organoleptic tests (relying on smell and sight), lactometers (for detecting possible adulteration), alcohol clot tests (indicating bacterial load and spoilage potential), and clot boiling methods (simple boiling to observe coagulation) (Tamanna, 2018).

The informal milk market in Kenyan cities and towns predominantly centers around low-income localities, driven by the affordability factor for consumers. As per the 2001 statistical abstract, around 60% of Nairobi's urban population inhabits low-income neighborhoods like Mathare, Kibera, Mukuru, Dandora, Kayole, Buru, Kangemi, Satellite, Umoja, Githurai, Kawangware, and Eastleigh. Raw milk stands as the most extensively consumed dairy product, notably utilized in tea and as fermented milk. Although there has been an increase in yogurt consumption as a snack, owing to the rise of small-scale dairy businesses, other dairy products are still trailing in terms of popularity (Mahmud, 2019).

2.3 Commercialization, Safety, Challenges and Economic Value of Milk in Kenya

Dairy production is of great importance to the economies and livelihoods of East Africans. In the region, Kenya stands out as the leading milk producer, with an annual production exceeding five billion liters. The dairy industry plays a substantial role in the nation's economy, contributing to about 40% of the livestock's gross domestic product (GDP), 14% of the agricultural GDP, and 3.5% of the overall GDP (Ajwang & Munyua, 2016). Smallholder dairy farmers are integral in this context, being responsible for producing roughly 75% of Kenya's total milk supply (Kashongwe *et al.*, 2017; Lelea *et al.*, 2023). Kenyans have one of the highest milk consumption rates in sub-Saharan Africa, consuming between 40 and 160 liters per capita per annum (Alonso *et al.*, 2018; Bosire *et al.*, 2017).

Elements like rapid population expansion, urbanization, and shifting culinary preferences among the middle class have collectively resulted in a steady annual rise of 5% in the demand for milk and its derivatives over the last ten years (Kabui *et al.*, 2015; Ondieki *et*

al., 2017; Wambugu *et al.*, 2011). In Kenya, the commercialization of milk occurs through both formal and informal value chains. The formal value chain is overseen by the Kenya Dairy Board (KDB) and encompasses entities licensed to pasteurize or apply ultra-heat treatment (UHT) to milk. These entities are responsible for packaging and marketing industrially processed dairy items such as "liquid milk," yogurt, and ice cream. On the other hand, the informal value chain, constituting the majority (70%) of milk trade in the country, involves the commercialization of raw and traditionally pasteurized milk and dairy products. This informal chain encompasses both licensed and unlicensed entities that directly sell milk and dairy products to consumers through outlets like milk bars, vending machines, corner shops, street vendors, and mobile vendors (Alonso *et al.*, 2018; Odero-Waitituh, 2017; Tuei *et al.*, 2021).

The assurance of milk safety and quality falls under the regulation of the Dairy Industry Act, enforced by the Kenya Dairy Board, as well as the Public Health Act, enforced by the Ministry of Health. The concept of milk quality pertains to attributes that contribute to the desirability of milk and its derivatives, while milk safety focuses on guaranteeing that its consumption poses minimal risk of harm or hazard. Despite this, a substantial portion of the milk traded within the informal value chain—primarily encompassing unprocessed milk—falls short of meeting the stipulated standards for composition and contamination established by regulatory authorities (Alonso *et al.*, 2018; Brown *et al.*, 2019).

Milk composition is subject to numerous influences including breed, age, health condition, diet, management methods, lactation phase, milking frequency, and environmental factors (Chen *et al*., 2014; Schwendel *et al*., 2015). These factors significantly impact the economic viability of processing and the caliber of dairy products. Research has highlighted instances of low protein content in certain studies scrutinizing milk composition in Kenya (Kabui *et al.*, 2015; Ondieki *et al.*, 2017).

Microbial contamination of milk transpires when bacteria from the cow's udder or its surroundings infiltrate the milk due to unsanitary milking and handling procedures. Both formal and informal value chains involve multiple actors handling milk, increasing the risk of microbial contamination. Cooling milk during bulking and transportation helps reduce microbial growth in the formal value chain, while the informal milk value chain lacks such measures (Kabui *et al.*, 2014; Nyokabi *et al.*, 2021). Bacterial contamination for instance with *Salmonella spp* and *Escherichia coli* poses public health risks to consumers in Kenya, and lactic acid bacteria contamination can lead to milk spoilage.

Several challenges are emerging in the field of detecting and determining antibiotic residues. A challenge lies in the rising count of novel substances accessible within the illicit market. These substances, exhibiting anabolic traits and employed for microbial management and growth enhancement, are uncovered annually. The realm of competitive sports illustrates the progression of such substances. Another notable concern involves the extensive habit of blending minute quantities of numerous substances. This practice results in a combined effect resembling the efficacy of employing a larger dose of a single substance, thereby complicating detection efforts. Additionally, the development of interfering substances to mask immunoassay detection systems further complicates the efficient detection of illegal substances. Control laboratories also face strict requirements for the performance of analytical methods based on new directives. This scenario presents obstacles for control laboratories owing to the considerable quantity and diversity of samples demanding analysis, the necessity to adjust analytical techniques to adhere to rigorous standards, escalated expenses linked to formulating fresh methodologies, the expanding roster of residues requiring scrutiny within each sample, and the imperative to invest in advanced instrumentation (Sachi *et al.*, 2019).

The presence of screening methodologies simplifies the oversight of chemical and veterinary substances in animal-derived food, curtailing the volume of samples necessitating resource-intensive and expensive confirmatory assessments. Modern advancements, presently obtainable in the market, are anticipated to be systematically adopted in the upcoming years, thereby broadening the screening of samples with heightened sensitivity. The enhancements in screening methodologies and their application will play a role in fortifying the safety assurance of animal-derived food (Ghernaout *et al.*, 2020).

2.4 Cattle Diseases and Antibiotics

Milk serves as an optimal breeding ground for bacteria, leading to various cattle infections and as a result driving to increased reliance on antibiotics. Antibiotics medications provide a remedy for bacterial contamination issues arising from pathogenic conditions in cattle. However, curing the animals can be challenging, potentially causing producers to administer excess medications, resulting in elevated concentrations of residues in milk. To address this, certain regulations stipulate the lowest maximum limits for veterinary drug residues, aiming to mitigate risks for consumers and prevent losses for producers (Bonomo, 2017)

The European Medicine Agency's (EMA) Committee for Medicinal Products for Veterinary Use (CVMP) is tasked with recommending Maximum Residue Limits (MRL) for animal-derived food products within the European Union (EU). The procedure is guided by Regulation (EC) 470/2009 of the European Parliament and the Council. Details concerning pharmacologically active substances and their categorization in relation to Maximum Residue Limits (MRL), particularly in products originating from animals, can be found in Commission Regulation (EU) No. 37/2010 dated 22 December 2009. While not as exhaustive as the EU Regulation, the Codex Alimentarius, endorsed by the Food and Agriculture Organization of the United Nations and the World Health Organization, also offers MRL information for different substances (Canzani & Aldeek, 2017).

2.4.1 Main causes of presence of antibiotic residues in milk

Availability of antibiotic residues (ARs) in milk can be attributed to a range of factors stemming from the use of antibiotics in various contexts. Therapeutic applications, where antibiotics are employed for treating infections like clinical mastitis and viral diseases, have led to the indiscriminate presence of ARs. Additionally, antibiotics are sometimes used as prophylactic measures during dry cow therapy and post-surgical risk management, contributing to the occurrence of ARs in milk. Furthermore, the application of antibiotics in diverse scenarios such as milk processing and preservation can indirectly or directly contaminate milk with drug residues (Canzani & Aldeek, 2017).

The adherence to label instructions plays a pivotal role in preventing residues in milk. Deviating from these guidelines can result in antibiotic residues being found in milk. This off-label use, involving the application of antibiotics meant for human use in animals, usage in non-approved species or conditions, or exceeding appropriate concentrations, contributes to the presence of residues in milk (Dettweiler *et al.*, 2020).

One crucial aspect is the maintenance of withdrawal periods, which refers to the time required for antibiotics to clear from an animal's system before its milk can be deemed safe for consumption. Inadequate adherence to withdrawal periods leads to elevated concentrations of ARs in milk. Insufficient detection facilities and monitoring systems, especially in developing countries, further exacerbate the issue. Regulatory organizations often lack the necessary strength to enforce proper monitoring and control of drug residues, resulting in subpar oversight (Dettweiler *et al.*, 2020; Enderle, 2012; FAO & WHO, 2018).

The impact of disease on animal metabolism can also intensify the presence of residues in milk. When animals are unwell, their metabolic processes can be disrupted, leading to the

extended storage of antibiotics in tissues and subsequently higher concentrations of residues. This underscores the importance of maintaining animal health for reducing the risk of drug residues in milk (Dettweiler *et al.*, 2020; Enderle, 2012; FAO & WHO, 2018).

Education and awareness are paramount in addressing the problem. Farmers' limited awareness of the potential health risks associated with ARs and inadequate education about proper antibiotic usage and withdrawal procedures contribute to the issue. Some of the farmers prefer treating the cows themselves without engaging veterinary doctors. This makes the use of drug residues rampant and unprofessional. Manufacturers' information also plays a role; incomplete or unclear literature from manufacturers can lead to improper usage and subsequent residue presence.

Practices related to equipment cleaning and disposal also impact AR presence. Inadequate cleaning of equipment contaminated with antibiotics and improper disposal of empty antibiotic containers on farms can lead to contamination of feeds and subsequent exposure of animals to ARs. Additionally, various factors influence the occurrence of ARs in milk. The type and concentration of antibiotics, use of excipients in medicine preparation, milking practices, udder tissue absorption, milk yield, and individual-specific factors are all pivotal elements in determining the presence of ARs. In light of these challenges, it is clear that tackling the issue of ARs in milk requires a multifaceted approach encompassing proper education, regulatory oversight, and improved practices in both antibiotic usage and milk processing (Felix *et al.*, 2021; Ferri *et al*., 2021).

2.4.2 Potential effects of Antibiotics Residues on the Dairy Sector and Public Health

Antibiotic Resistance has been reported to be a major problem. The emergence of antibiotic resistance is intricately linked to the presence of trace amounts of antibiotics in dairy products, including milk. These minute residues create an environment in which microorganisms can adapt and become resistant to the very drugs meant to combat them. This poses a grave risk as these antibiotic-resistant strains can be transmitted directly through contact or indirectly by sharing resistance genes with other bacteria in the surroundings. This phenomenon has far-reaching implications, potentially leading to infections that are extremely challenging to treat effectively (Dettweiler *et al.*, 2020; Felix *et al.*, 2021; Ferri *et al*., 2021).

Allergic Reactions is another effect. The residues of diverse antibiotics found in dairy products are associated with a range of allergic reactions, notably including conditions like serum sickness and anaphylaxis, particularly prominent in the case of penicillin. These

allergic responses can trigger severe health complications among individuals consuming these products. The presence of such allergenic residues underscores the significance of stringent measures to avoid their occurrence in dairy items (Dettweiler *et al.*, 2020; Felix *et al.*, 2021; Ferri *et al*., 2021).

Another issue is carcinogenicity. An unsettling consequence of antibiotic residues is their potential to incite carcinogenic effects. These residues can interact with cellular components such as DNA and RNA, potentially inducing changes that contribute to the development of cancerous cells. This underscores the urgency of preventing the presence of these residues in dairy products, as they may inadvertently expose consumers to potential cancer risks ((Dettweiler *et al.*, 2020; Felix *et al.*, 2021; Ferri *et al*., 2021).

Mutagenicity effect has also been associated with antibiotics**.** Antibiotic residues have the troubling potential to act as mutagens, inducing mutations in DNA molecules or causing damage to chromosomes. Such genetic alterations can lead to a cascade of negative impacts, potentially even impacting fertility and reproductive health in humans. The mutagenic nature of these residues highlights the need for robust strategies to minimize their presence in dairy products ((Dettweiler *et al.*, 2020; Felix *et al.*, 2021; Ferri *et al*., 2021).

Teratogenicity is an important factor to also consider. Prolonged exposure to antibiotic residues, particularly during the crucial gestation period, can result in various congenital anomalies in newborns. This underscores the imperative of preventing antibiotic residues moving into the dairy supply chain to ensure health and well-being of both mothers and infants (Gussmann *et al*., 2017).

Disruption of Normal Intestinal Environment is another effect. The presence of antibiotic residues in dairy products, originating from the usage of broad-spectrum antibiotics, can perturb the balance of the normal intestinal microflora. Beyond targeting harmful microorganisms, these residues can inadvertently impact beneficial ones, potentially creating an environment that favors the proliferation of disease-causing bacteria. This disruption has implications for gut health and overall well-being (Dettweiler *et al.*, 2020; Gussmann *et al*., 2017). Moreover, Impact on Dairy Industry should also be considered. The effects of antibiotic residues extend beyond health concerns, influencing the processes of the dairy industry. Even minuscule concentrations of these residues can interfere with the intricate fermentation processes which are crucial for producing dairy products like cheese and yogurt. Such interference can compromise the quality and safety of these products, prompting the need for stringent monitoring and preventive measures (Dettweiler *et al.*, 2020; Gussmann *et al*., 2017).

These concerns underscore the necessity of rigorous monitoring, regulatory oversight, and proactive measures to mitigate the presence of antibiotic residues in dairy products. It is not only about safeguarding consumer health but also ensuring the sustainability and integrity of the dairy industry while mitigating potential risks to the environment. The convergence of health, economic, and environmental interests calls for collaborative efforts from all stakeholders to address this complex challenge effectively (Dettweiler *et al.*, 2020; Gussmann *et al*., 2017).

2.4.3 Prevention and Control Measures Reducing ARs in Milk

Antibiotic Resistance in Dairy Products is a factor to be seriously considered during the preventive measures. The presence of trace amounts of antibiotics in dairy products, particularly milk, has become intricately linked to the concerning rise in antibiotic resistance. These minute residues create an environment in which microorganisms can adapt and develop resistance to the antibiotics designed to counteract them. This scenario poses a serious risk as these antibiotic-resistant strains can be transmitted directly through contact or indirectly by sharing their resistance genes with other bacteria in the surrounding environment. This phenomenon carries profound implications, potentially leading to infections that are notably challenging effective treatment. Addressing this issue requires concerted efforts to prevent the entry of antibiotic residues into dairy products, safeguarding public health (Dettweiler *et al.*, 2020; Gussmann *et al*., 2017).

Additionally, allergic reactions and beyond require attention. A range of allergic reactions, encompassing conditions like serum sickness and anaphylaxis, has been linked to the presence of various antibiotic residues within dairy products. This connection is especially pronounced in instances involving penicillin residues. These allergic responses can trigger severe health complications in individuals consuming such products. Recognizing the presence of these allergenic residues underlines the importance of stringent measures to eliminate their occurrence in dairy products (Dettweiler *et al.*, 2020; Hawkey & Livermore, 2012).

Control of Potential Carcinogenic and Mutagenic Effects needs also pragmatic approach. Antibiotic residues also pose potential carcinogenic and mutagenic effects. These residues can interact with cellular components like DNA and RNA, potentially inducing changes that contribute to the development of cancerous cells or genetic mutations. The presence of these residues thus introduces potential cancer risks to consumers, highlighting the urgency of preventing their occurrence in dairy products to ensure consumer safety.

Implications for Newborns and Intestinal Health is another factor. Extended exposure to antibiotic residues, especially during the critical gestation period, can lead to various congenital anomalies in newborns. This give emphasis to the significance of excluding antibiotic residues from the dairy supply chain to safeguard the health of both mothers and infants. Additionally, the presence of antibiotic residues originating from broad-spectrum antibiotics can disrupt the delicate balance of normal intestinal microflora. This imbalance can extend beyond targeting harmful microorganisms, inadvertently impacting beneficial ones. This disruption holds implications for gut health and overall well-being (Dettweiler *et al.*, 2020; Herreros *et al.*, 2005).

Industry Impact and Mitigations should be highly considered**.** The repercussions of antibiotic residues extend beyond health concerns, significantly affecting dairy industry processes. Even minute concentrations of these residues can interfere with intricate fermentation processes crucial for producing dairy products such as cheese and yogurt. This interference jeopardizes product quality and safety, necessitating rigorous monitoring and preventative measures (Dettweiler *et al.*, 2020; Herreros *et al.*, 2005).

These concerns emphasize the pressing need for rigorous monitoring, regulatory oversight, and proactive approaches to mitigate the presence of antibiotic residues in dairy products. The imperative extends beyond safeguarding consumer health, encompassing the sustainability and integrity of the dairy industry while minimizing potential environmental risks. A collaborative effort involving stakeholders from various sectors is essential to effectively address this complex challenge (Dettweiler *et al.*, 2020; Herreros *et al.*, 2005). Appropriately addressing antibiotic residues involves comprehensive strategies which include: Sensitive Detection and Quantification: to counteract false-negative results, it's vital to develop highly sensitive detection methods. Employing approaches that ensure minimal chances of false-positive outcomes is equally crucial. Another control includes regular monitoring in which it includes implementing nationwide monitoring policies is crucial to consistently assess the concentration of antibiotic residues in milk (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020). Moreover, inactivation techniques can be used: Some antibiotics can be rendered inactive through measures such as refrigeration, pasteurization, UV radiation, activated charcoal, or resin treatments. Another way is public awareness. Effective awareness campaigns led by experts can educate the public about the implications of antibiotic residues and encourage responsible consumption. Additionally, restricting veterinary antibiotics can reduce the effect. Prohibiting indiscriminate veterinary antibiotic usage is pivotal to curbing antibiotic residues (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020).

Another method is through Exploring Herbal Alternatives: Herbal sources of medicines could serve as alternative options for treating diseases, reducing reliance on antibiotics. Also, effective drug use program can be implemented. Adhering to guidelines is essential, including proper withdrawal times, reading label instructions, avoiding intermixing of drugs, maintaining hygiene during administration, and implementing biosecurity measures. Farm Management Practices is another method applied. Prioritizing animal health, adopting better biosecurity practices, proper identification of treated cows, and meticulous record-keeping are fundamental. Also, use of segregation and discard. Implementing processes to separate and withhold milk from antibiotic-treated cows can minimize the risk of antibiotic residues. Last but not least, is Education for Dairy Producers: Empowering dairy producers with knowledge about milk quality assurance is vital to ensure the production of safe dairy products. These multi-faceted strategies emphasize the significance of proactive measures, collaboration among stakeholders, and a comprehensive approach to address the challenges posed by antibiotic residues in dairy products (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020).

Currently, availability of antibiotic residues (ARs) in products of milk has evolved into a critical and multifaceted concern, capturing significant attention due to its profound implications for public health. Research findings have illuminated the diverse array of factors contributing to the emergence of ARs in milk. These causative agents are not limited in number; rather, a multitude of factors collectively contribute to the availability of ARs in milk and milk prodcts. Achieving accurate detection and quantification of these residues in a cost-effective and timely manner remains an ongoing challenge (Kastrinos, & Weber, 2020).

Recent advancements have yielded a range of techniques aimed at detecting antibiotic residues, and the research landscape continues to evolve as investigators strive to refine these methods. Amidst this pursuit, chromatographic techniques have emerged as the linchpin, offering unparalleled sensitivity, specificity, reliability, and feasibility in the context of modern requirements. Notably, various adaptations of chromatographic methods are currently under exploration, underscoring the need for comprehensive future research endeavors to unlock their maximal potential (Kastrinos, & Weber, 2020).

The escalating momentum of research activities in this domain serves as a telling indicator of the escalating utilization of antibiotics in livestock. This trend simultaneously highlights the looming threat of antibiotic residues and engenders mounting concern among stakeholders. In light of this, it becomes increasingly imperative to enact effective measures that curtail the presence of ARs in milk. Addressing this issue necessitates a multi-pronged

approach, encompassing stringent regulations, improved veterinary practices, heightened awareness among farmers, and enhanced monitoring techniques. Collaborative efforts involving regulatory bodies, agricultural practitioners, research institutions, and public health authorities are indispensable for mitigating the proliferation of ARs in milk (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020).

Ultimately, the recognition of the multifaceted nature of this challenge highlights the urgency of comprehensive action. Striving to eliminate ARs from milk not only safeguards public health but also upholds the integrity of the dairy industry while aligning with sustainable practices. By employing a holistic strategy that embraces innovative technologies, regulatory oversight, and a commitment to responsible antibiotic usage, we can pave the way for a safer and healthier dairy landscape (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020).

2.4.4 Smallholder Dairy Improvement and Opportunities

The growing desire for dairy and milk products presents lucrative market prospects for domestic production. Fresh milk can be marketed to both urban residents in densely populated lower-income areas and milk-deficient rural regions, thanks to the affordability factor for consumers. Moreover, the rise in disposable incomes and evolving consumer preferences in urban areas have driven domestic demand for premium food items like milk and its derivatives. The substantial regional markets arising from regional integration initiatives and preferential treatment for member countries hold significant potential for elevating smallholder dairy production and marketing. Capitalizing on emerging and existing dairy and milk product markets can result in expanded trade and augmented earnings for both the nation and the broader region. However, effectively harnessing these opportunities necessitates addressing challenges linked to enhancing safety and quality, boosting efficiency, and fortifying the competitive edge in milk and dairy product production and marketing (Odero-Waitituh, 2017).

Expanding and enhancing the current road network is essential to lowering dairy production costs and boosting the quantity of marketed milk. The focus should be on upgrading feeder roads that connect farms to milk collection centers, especially those that become impassable during the rainy season. Enhancing the collection and marketing of milk from farms is vital, considering the perishable nature of milk and its products. Furthermore, an expanded and improved road network would facilitate the transportation of inputs and other dairy production support services. Both the central government and local communities,

through innovative partnerships including collaborations with the private sector, can contribute to the improvement and expansion of the road network (Odero-Waitituh, 2017).

Marketing developments within the smallholder dairy sector illustrate that the informal segment, constituting around 64% of the marketed milk, primarily deals with raw milk, commonly consumed in tea, coffee, or as a food snack. Consumer preference for raw milk over processed alternatives confers a competitive edge to both the informal sector and the smallholder dairy production system. The utilization of the Lacto peroxidase System (LPS), recommended by the Food and Agriculture Organization, as a means of preserving raw milk, presents a safe approach that can be adopted in cases where cooling facilities are either unavailable or unaffordable. However, the establishment of policies supportive of LPS adoption for milk preservation is necessary (Rademaker *et al*., 2016).

Marketing developments within the smallholder dairy sector illustrate that the informal segment, constituting around 65% of the marketed milk, primarily deals with raw milk, commonly consumed in tea, coffee, or as a food snack. Consumer preference for raw milk over processed alternatives confers a competitive edge to both the informal sector and the smallholder dairy production system. The utilization of the Lacto peroxidase System (LPS), recommended by the Food and Agriculture Organization, as a means of preserving raw milk, presents a safe approach that can be adopted in cases where cooling facilities are either unavailable or unaffordable. However, the establishment of policies supportive of LPS adoption for milk preservation is necessary (Kilelu *et al.,* 2017).

Improving access to support services and technologies is crucial for dairy production. Farmer cooperatives, self-help groups, private processors, and other partners can play a role in providing support services. An improved and expanded road network would enhance farmers' access to important dairy technologies and production services. Employing artificial insemination (AI), extension services, animal health support, training, and credit facilities can amplify the adoption of contemporary agricultural inputs and suitable production technologies, ultimately elevating dairy productivity. It's vital to foster heightened community and private sector engagement to complement governmental endeavors in delivering support services and technologies to farmers. Nevertheless, prioritizing coordination and engagement from essential stakeholders is crucial, guaranteeing effective management, accountability, and investments aligned with the farmers' welfare (Odero-Waitituh, 2017; Kilelu *et al.,* 2017).

For sustainable use of natural resources, it is necessary to enforce legislation that prohibits wetlands, water catchment, animal/crop production in steep areas, deforestation,

human settlement and over-grazing. Establishing fodder trees and legumes can improve soil fertility and increase biomass yield, providing quality forage for dairy cattle. Collaborative efforts and partnerships with relevant stakeholders are essential for the sustainable protection of the environment and biodiversity conservation while utilizing natural resources in these fragile environments (Ter-Hemen, 2015).

2.5 Control of Livestock Diseases Using Antibiotics and their Effects

The most significant contribution to sustainable development aimed at reducing poverty lies in the improvement of livestock production systems. Livestock enterprises are important in supporting the livelihoods of small-scale farmers, consumers and traders especially in the developing world. Animal diseases are crucial constraints because of their high occurrences and the costs related to their treatment. Illustratively, as per Nyaguthii (2019), the global distribution of foot-and-mouth disease (FMD) mirrors poverty indicators closely. In this context, small-scale farmers possess limited livestock and resources to withstand adverse impacts. Consequently, the loss of individual animals bears immense significance for their livelihoods (Rapsomanikis, 2015).

The transmission, impacts, and management of animal diseases manifest through diverse mechanisms. The principal disease groups are categorized into epidemic diseases, which encompass ailments like swine fevers, Newcastle disease (ND), rinderpest and Foot and Mouth Disease. These diseases pose a significant threat to national livestock sectors due to direct repercussions like elevated morbidity and mortality rates, substantial expenses linked to control or eradication programs, and limitations on livestock and livestock product trade. The influence of these diseases extends to livestock producers, individuals working within the livestock industries, and consumers alike (Perry & Grace, 2009). Zoonotic diseases, including rabies, bovine spongiform encephalopathy (BSE), Rift Valley fever (RVF), hydatid disease and brucellosis can exert impacts primarily on human health, animal health, or both. These diseases tend to have the most pronounced effects on individuals closely associated with livestock, such as livestock keepers residing near their animals, butchers, and other workers engaged in handling livestock products (Fevre *et al.,* 2017). Foodborne infections and intoxications, exemplified by pathogens like *Salmonellosis* and *Escherichia coli* O157, pose specific challenges within more automated industrial systems. The likelihood of their occurrence is anticipated to rise in tandem with the intensification of livestock production and processing systems. As highlighted by Nespolo (2021), food-borne diseases exert their influence on a wide spectrum of stakeholders including consumers, food

processing personnel, and livestock producers. The presence of endemic diseases like mastitis and pneumonia, alongside parasitic afflictions such as trypanosomosis and helminthosis, engenders repercussions for both livestock enterprises and consumers. These repercussions manifest in the form of diminished productivity, augmented control expenditures, and indirect losses tied to compromised nutrition and other constraints inherent to livestock production. Broader economic impacts resulting from endemic diseases are trade restrictions in livestock and livestock products and reduced capacity for value-added trade (Hashem *et al.*, 2020).

Animal diseases have direct implications on livestock productivity, leading to diminished feed consumption, alterations in digestion and metabolic processes, heightened levels of sickness and death, and lowered rates of reproduction, weight increase, and milk yield (Temple & Manteca, 2020). These cumulative consequences curtail critical decisions related to economically significant herd management, encompassing choices about animal selection and the most suitable lifespan (Rojas-Downing *et al.*, 2017).

The interplay among disease, nutrition, and genetic selection underscores the necessity of addressing the repercussions of both epidemic and endemic diseases prior to realizing the potential benefits of improved nutrition and genetic initiatives (Rohr *et al.*, 2019). Merely focusing on disease control may not guarantee significant gains in productivity and economics. The utilization of antibiotics is deemed essential to maintain optimal animal health and achieve growth or production efficiency. The necessity for therapeutic applications becomes evident when confronted with the potential losses that can arise from the resurgence of active infection and disease within a herd (Saad & Ahmed, 2018). Despite the predominant usage of antibiotics within agricultural environments, there has been limited focus on understanding how antibiotic use in farm animals contributes to the broader issue of antibiotic resistance. Apprehensions regarding the connection between antibiotic usage in food animals and the emergence of drug resistance in pathogens, animals, and, ultimately, humans have triggered endeavors to curtail the application of antibiotics in animal production (Ma *et al.*, 2021) whenever feasible. Diminishing the utilization of antibiotics in food animals should yield advantages for both human and animal health by lowering the occurrence and intensity of diseases. Approaches aimed at curtailing the extent of therapeutic antibiotic application are contingent upon the capability to preempt diseases and infections, substantiated identification of pathogen presence through diagnosis, and the selection of antibiotics that are potent and comprehensive in eradicating infections (Ayukekbong *et al.*, 2017). To halt the cycle of repeated trial-and-error antibiotic usage, it is imperative that the

bacteria exhibit susceptibility to the prescribed antibiotic. Furthermore, it is essential to differentiate between viral diseases and bacterial diseases to avoid confusion (Worthington & Melander, 2013).

Reducing the use of antibiotics for sub-therapeutic disease prevention and growth promotion in food animals presents a significant opportunity for decreasing overall antibiotic usage (Dwivedi *et al.,* 2017). Alternative strategies, such as implementing effective management practices, can play a crucial role in achieving this goal. Good agricultural practices that optimize the genetic growth and productivity of food-producing animals, as well as provide appropriate and timely nutrition, can help prevent physiological strains that may compromise overall animal health (Dwivedi *et al.,* 2017).

While it may not be possible to completely eliminate the need for antibiotic use in food animals, employing strategies that promote judicious and prudent antibiotic usage can have a positive impact on the animal industry (WHO, 2017). By integrating approaches that rely less on antibiotics, it becomes feasible to reduce the incidence of diseases. When disease does arise, strategic and suitable antibiotic usage can diminish the duration and seriousness of the illness. Moreover, bolstering animals' immune capabilities can augment the efficacy of therapeutic interventions when antibiotics become essential. This approach not only aids in mitigating the negative repercussions of antibiotic excess but also guarantees elevated levels of animal well-being, food quality and production (Ayukekbong *et al.,* 2017).

To reduce disease incidence and subsequently decrease the reliance on antibiotics in food animals, various management strategies and preventive medicine programs can be implemented. These measures include implementing strict controls on hygiene, population dynamics, feed quality, and environmental conditions to prevent or minimize stress. Disease eradication can be achieved through vaccination programs, while optimal nutrition can enhance natural immunity and buffer animals against sudden changes in conditions. Additionally, selective breeding for genetically disease-resistant livestock and exploring alternative growth promoters with negligible residue issues are viable options (World Health WHO, 2017). Although disease eradication efforts may involve initial costs, they can be economically justified, especially when there is a significant public health risk.

2.5.1 Livestock Products and Presence of Antibiotic Residues

Antibiotics, comprising naturally-occurring, semi-synthetic, and synthetic compounds endowed with antimicrobial attributes, are regularly administered via oral, parenteral, or topical routes in both human and veterinary medical practices. They serve purposes such as

treating and preventing diseases, as well as promoting growth in food animals (Oyebanji, 2018). Antibiotics are extensively utilized in livestock farming on a global scale, yet the destiny of their residues and their potential ramifications on environmental well-being frequently remain uncertain. Inappropriate antibiotic practices, encompassing their application for purposes such as enhancing performance and feed efficiency, regulating reproductive cycles, and bolstering breeding performance, have resulted in a surge of diseases among humans and domestic animals across the world (Bett *et al.*, 2020).

The utilization of antibiotics in animals can lead to effects on human health, both directly and indirectly. Direct effects entail the connection between exposure to antibiotic-resistant bacteria originating from food animals and resulting health concerns. Conversely, indirect effects arise from contact with resistant organisms that have disseminated throughout various elements of the ecosystem, like water and soil, due to the use of antibiotics in food animals (Marshall & Levy, 2011). Concerns about antibiotic residues in animal-derived food arise from the potential direct humans' toxicity and the possibility of low-level antibiotic exposure leading to alterations in the microflora, disease development, and the emergence of resistant strains that can undermine antibiotic therapy.

The presence of antibiotics utilized in food animals can introduce health risks due to their trace amounts found in edible animal tissues. Certain medications carry the potential to induce direct toxic reactions or trigger allergic/hypersensitivity responses in consumers (Tariq *et al.*, 2020). For example, beta-lactam antibiotics, including penicillin and cephalosporin groups, can lead to anaphylaxis, gastrointestinal symptoms, dermatitis and cutaneous eruptions even at low dosages (Felix *et al.*, 2021). These direct effects may include the development of antibiotic resistance in the normal flora of the human gastrointestinal tract through the consumption of meat products containing antibiotics, resulting in outbreaks of resistant diarrheal diseases. Furthermore, there is an elevated risk of developing antibiotic-resistant colonization or infections in humans as a consequence of contact with farm animals subjected to antibiotic treatment. Indirect and prolonged risks encompass a range of microbiological consequences, along with the potential for carcinogenicity, reproductive effects, and teratogenicity (Fitzpatrick *et al.*, 2017). Microbiological effects, in particular, pose a significant health hazard to humans. Resistant bacteria present in animal waste used as fertilizer can contaminate water supplies and alter human flora (Manyi-Loh *et al.*, 2018).

2.5.2 Maximum Residue Limits (MRL)

The maximum residue level (MRL) refers to the highest concentration or level of a drug or chemical that is considered non-hazardous and permitted by regulatory bodies in food or feed intended for animal or human consumption at a specific point in time. The unit used to express this maximum allowable concentration is milligrams per kilogram for solid products and milligrams per liter for liquids (Sachi *et al.*, 2019; Zikankuba *et al.*, 2019).

When milk and other dairy products contain drug residues that exceed the MRL, it can lead to significant health problems for consumers. Although the availability of high-quality milk and associated products is crucial for safeguarding public health, the inclusion of antibiotic residues in these food items, if ingested, can potentially lead to health implications including cancer, hypersensitivity reactions, and the emergence of antibiotic resistances. The consequences of antibiotic resistance are particularly concerning, as it renders antibiotics clinically ineffective. Adhering to proper withdrawal times, specifically established for milk and other food products, can serve as a safeguard to prevent the harmful effects of antibiotic residues (Nowak & Nowak, 2021).

2.6 Udder infections, Control and Antimicrobial Resistance by the Mastitis Microbes

2.6.1 Impact of Udder Infection on Dairy Industry

Mastitis refers to the inflammation of udder tissue and mammary glands in cows, predominantly triggered by bacterial infections. It can result in partial or complete damage to the udder, leading to significant economic losses for farmers (Abdalhamed *et al.*, 2018). While mastitis cannot be completely eradicated, it can be reduced to low levels through proper management of dairy cows. Bacterial infections are the primary cause of mastitis, although other microorganisms such as fungi, yeasts, and viruses can also contribute (Tarazona-Manrique *et al.*, 2019). The most common pathogens involved in mastitis are *Escherichia coli, Streptococcus uberis, Streptococcus dysgalactiae*, *Streptococcus agalactiae* and *Staphylococcus aureus*, although other pathogens can occasionally cause outbreaks in herds (Gao *et al.*, 2017; Suojala *et al.*, 2013).

The severity and treatment of mastitis depend on the individual case. Not all cases require antibiotic treatment since some can be managed effectively without it (Roberson, 2012). *Escherichia coli*-induced mastitis can manifest as a spectrum of subclinical infections that go unnoticed to systemic infections with severe symptoms (Suojala *et al.*, 2013). In cases of severe clinical mastitis, antibiotics are typically necessary along with other treatments aimed at reducing inflammation. Mastitis occurs when pathogens enter the teat duct and

initiate an infection in one or more quarters of the udder. The cause of infection can vary, with some cases persisting for weeks or months in a mild form (subclinical mastitis) that may go undetected in milk production. Infections caused by certain pathogens like *E. coli* can be more acute, leading to general endotoxemia with elevated body temperature, loss of appetite, and potential fatality if not treated promptly (Nickerson & Ryman, 2019).

Clinical mastitis is effectively treated with antibiotic infusions through the teat duct, and the interventions generally lead to the resolution of clinical symptoms and frequently result in the elimination of the bacterial infection. While some infections may spontaneously recover, many persist and require antibiotic therapy or cow culling for eventual elimination. The susceptibility to mastitis varies among cows,and new infections are most prevalent among older cows at the onset of early lactation, as well as during the initiation of the dry period and periods of inadequate management practices (Niemi *et al.*, 2021).

Mastitis leads to direct economic losses for farmers in several ways. Reduced milk yields occur when abnormal or antibiotic-contaminated milk cannot be sold for consumption. Additional costs include veterinary care, antibiotic expenses, higher culling rates, and occasional fatalities. The magnitude of these effects depends on factors such as the virulence of the causative pathogen, the persistence of the infection into lactation, and the timing of infection. Prolonged infections during lactation can have a significant impact on the future udder health and milk production of affected cows (Nitz *et al.*, 2020).

Antibiotic therapy continues to play a vital role in enhancing animal production and managing mastitis in dairy cows, though its usage should be exercised with prudence. The primary objective of mastitis control is the prevention of new infections, even though occurrences of new cases are inevitable. Once an infection takes hold, strategies for eradicating the disease comprise spontaneous recovery, the removal of chronically infected cows, treatment during lactation, and the implementation of dry cow therapy. Antibiotic treatment stands as the primary method for addressing cases of mastitis and constitutes a primary rationale for the use of antibiotics in dairy cows (Kromker & Leimbach, 2017).

Successful antibiotic therapy requires drugs to reach all infection sites within the affected quarter, maintain adequate levels at those sites for a sufficient duration, and effectively kill all infecting microorganisms (Niemi *et al.*, 2021). With the exception of *E. coli* infections, most microbial infections persist until spontaneous recovery or cow culling. This persistence of infection for weeks, months, or years, particularly with *staphylococcal* infections, is a crucial characteristic of mastitis that must be considered when developing disease control strategies. Antibiotic therapy represented a significant advancement in

controlling bovine mastitis, as spontaneous recovery is infrequent and increasing it has proven challenging (Nickerson & Ryman, 2019).

Antimicrobial drugs used in mastitis therapy include penicillin and its derivatives, streptomycin, and aureomycin, among others (Kumari *et al.*, 2018). The effectiveness of a drug depends on the sensitivity of the targeted pathogen and the formulation of the drug, which influences its excretion, metabolism, distribution and absorption from milk. There is no ideal antimicrobial drug suitable for all conditions, and mixtures are often used to address a range of pathogens in lactating and dry cows. In most cases, treatment is initiated without prior knowledge of the specific causative pathogen. Typical products employed for addressing clinical mastitis attain bacteriological eradication rates of 75-90% for streptococcal infections and approximately 30% for staphylococcal infections. Cure rates for mycoplasma and *Pseudomonas* infections are generally lower. Dry cow therapy, utilizing formulations tailored for this purpose, enhances cure rates and achieves an approximately 50% elimination of *staphylococcal* infections. Nonetheless, the rates of elimination for staphylococcal infections can exhibit significant variation between herds and are reduced in cows with multiple affected quarters and more severe instances of clinical mastitis. (Abebe *et al.*, 2016).

During antibiotic therapy, it is important to clean the teat orifice carefully with a disinfectant swab and use sterile disposable syringes to avoid introducing contaminants. Milk from treated cows should not be supplied for at least two or more days after the last infusion to prevent antibiotic contamination of the bulk milk. Although most cases of clinical mastitis respond quickly to treatment, it may take several days for the milk to return to normal. Decisions regarding milk discard time, choice of antibiotic and treatment should be based on veterinary advice. For severe and enduring infections, the systemic administration of antibiotics may become necessary to address endotoxemia (Nickerson & Ryman, 2019). Culling cows is an effective method of eliminating many infections, and increasing the sale of cows with persistent clinical mastitis can further contribute to control efforts.

2.6.2 Types of Antibiotics used in Controlling Udder Infection and their Relationships

Antibiotics play a crucial role in managing herd health, particularly in preventing and controlling diseases that affect milk producing cows, such as reproductive tract infections and mastitis (Asredie & Engdaw, 2015). These diseases can be effectively treated and potentially controlled with the use of antibiotics. Antibiotics are administered to animals through various routes, including injections, oral administration, topical application, intramammary infusions,

and intrauterine infusions. Among these methods, the frequency of antibiotic residues in milk is lower after intrauterine infusion compared to other routes of administration (Zhang *et al.,* 2015).

Globally, the most commonly used antibiotics in the dairy industry belong to five major classes: tetracyclines (14.01%), beta-lactams (36.54%), fluoroquinolones (13.46%), aminoglycosides (10.44%) and sulfonamides (12.64%) (Sachi *et al.,* 2019). The maximum residue limits (MRLs) for these antibiotics are specified in micrograms per kilogram: 4 for beta-lactams, 100 for tetracyclines, 200 for fluoroquinolones, 100 for sulfonamides, and 100 for aminoglycosides (Ali *et al.,* 2017).

When it comes to managing bovine mastitis, different antibiotics are used depending on the causative agents. For *E. coli*-related mastitis, fluoroquinolones and cephalosporins are commonly employed (Suojala *et al.,* 2013). *Staphylococcu*s-related mastitis is often treated with intramammary pirlimycin, while *A. pyogenes*-related mastitis may be addressed with florfenicol, ceftiofur, or penicillin (Roberson, 2012). Intramammary antibiotics have shown the highest effectiveness in mastitis treatment. However, it is crucial to adhere to the recommended withdrawal periods, which are as follows: 72 hours for penicillin, 48 hours for cloxacillin, 36 hours for pirlimycin, 60 hours for amoxicillin, and 96 hours for cephapirin (Pyorala *et al.,* 2009). During mastitis treatment, drugs are typically administered through infusions and injections for three consecutive days to ensure better results. Consequently, the withdrawal period extends to six days, including three days of treatment and an additional three days to observe the withdrawal period. This extended withdrawal period may contribute to the presence of residues in raw milk, resulting in losses for farmers.

Mastitis leads to an increase in somatic cell counts (SCC) in the milk from infected quarters. High SCC is often indicative of mastitis and influences farmers' decisions to administer antimicrobial substances. As a result, high SCC values may coincide with an increase in antibiotic residues in milk, as reported by Mahmoudi *et al*. (2013) and Gussmann *et al.* (2017). Treatment and prevention of mastitis typically involve the use of antimicrobial drugs, and some farmers fail to observe the recommended withdrawal periods (Kunda *et al.,* 2016). Mahmoudi *et al.* (2013) conducted a study to examine the relationship between SCC and the risk of antibiotic residue violations, and their findings showed significantly higher SCC values in samples that tested positive for antibiotic residues in milk. Furthermore, the rate of antibiotic residue violations increased with higher SCC levels. Consequently, as farmers perceive high somatic cell counts as an indication of mastitis, antibiotic violations often occur as they attempt to combat the effects of the disease using antibiotics.

Inappropriately or unnecessarily treating nearly 50% of clinical mastitis cases with antibiotics has been reported (Roberson, 2012).

2.6.3 Antimicrobial Resistance and the Mechanisms in their Resistance

Antimicrobial resistance (AMR) is a pressing global issue with potential risks associated with water, sanitation, and hygiene factors (Fletcher, 2015). In the environment, antimicrobial resistance naturally develops among microorganisms as part of an evolutionary process. It is believed that antibiotic resistance genes originate from environmental bacteria that produce and release antibiotics to influence competing microbial populations (Davies & Davies, 2010). These genes can be found in self-transferable plasmids or transposable elements, allowing for genetic transfer between related and unrelated bacteria. Some organisms release antibiotics into their environment to control competing bacteria, and in this process, naturally occurring resistance genes may be captured by mobile genetic elements, leading to the introduction of resistance genes into clinically relevant bacterial species from environmental sources (Peterson & Kaur, 2018).

It's crucial to recognize that resistance, whether naturally occurring or acquired, can be transmitted both horizontally and vertically. Similar resistance genes have been identified in unrelated pathogens across diverse environments like humans, clinical environments, agriculture, meat animals, and companion animals. This implies that antimicrobial resistance is not contingent on context (Fletcher, 2015). Nevertheless, the selective pressure stemming from widespread and frequent antibiotic utilization can expedite the development of resistance. While intrinsic resistance is relatively uncommon, acquired resistance under pressure is more widespread (Munita & Arias, 2016). Certain bacterial resistance mechanisms have the potential to transfer to humans, leading to an initial latent carrier state that could subsequently give rise to seemingly unconnected infections and heightened disease severity in patients. Grasping the environmental factors that contribute to the prevalence of resistant pathogens is of paramount importance, as it enables the identification of modifiable interactions and facilitates efforts to curb the further propagation of resistance (Fletcher, 2015).

Different microorganisms employ various mechanisms to resist antibiotics, including enzymatic degradation or modification, target modification, changes in cell wall permeability, efflux pumps, and deviation from normal physiological pathways (Smith *et al.*, 2015). Enzymatic degradation or modification involves the breakdown of antibiotics by enzymes like beta-lactamases and acetyltransferases. Target modification refers to altering

the target molecule of the antibiotic, making it ineffective. Changes in cell wall permeability can reduce antibiotic entry or increase efflux, regulating the internal antibiotic concentration. Deviating from normal physiological pathways involves the inclusion of alternative steps facilitated by additional enzymes. Specific environmental issues contributing to antimicrobial resistance include animal husbandry, soil-borne resistance, horizontal gene transfer and waste management, food safety, wastewater and drinking water (Ondon *et al.,* 2021).

Antimicrobial Resistance (AMR) represents a worldwide menace to human health, agriculture, and ecosystems. A multi-sectoral and global approach is necessary to mitigate the spread of antibiotic resistance worldwide. However, some countries have not adequately addressed and investigated antibiotic resistance issues. A substantial knowledge gap exists concerning the dissemination of antibiotic resistance within the agriculture and dairy sectors, both of which hold pivotal roles in the production of animal-derived food. Studies have highlighted outdated practices and lack of control over veterinary medicine sales and usage in the Georgian dairy sector, posing food safety risks (Khakhviashvili *et al.*, 2020). Studies conducted in the domain of human health have highlighted the emergence of antibiotic resistance in *Staphylococcus aureus*, a prevalent cause of bovine mastitis. However, antibiotic resistance within the context of dairy farming remains insufficiently investigated. Monitoring findings suggest a heightened likelihood of antibiotic-resistant bacterial strains evolving in dairy farming and subsequently propagating through the environment via the food chain (Sachi *et al.*, 2019).

Antibiotic usage in animals, including dairy cattle, has exacerbated the evolution of strains that are resistant. The misuse and overuse of antibiotics for controlling mastitis in dairy cattle have implications for antimicrobial resistance. Studies from different countries have shown varying percentages of antibiotic-resistant *S. aureus* isolated from milk samples (Khakhviashvili *et al.*, 2020). Resistant *Staphylococcus strains* can spread through the food chain to the environment, increasing the risk to biodiversity and human health. Bidirectional transmission of *S. aureus* strains between humans and livestock is common. Methicillin-Resistant *S. aureus (MRSA*) has become a significant threat globally, with a suspected origin in dairy cattle (CC97 clone). However, the risks associated with AMR spreading through the food chain and environment remain unaddressed, and current practices in dairy farming are not adequately reviewed for their effects on AMR development (Khakhviashvili *et al.*, 2020).

2.7 Challenges Related to Milk Quality and Processing due to Antibiotics

2.7.1 Milk Quality Challenges Related to Antibiotic Residues

Antibiotic residues in animal-derived food products, such as milk, eggs, and meat, are remnants of antimicrobial substances used in animal treatment. The presence of these residues has become a significant concern worldwide due to the increased use of antibiotics for treating animals and promoting growth. Several studies have reported the presence of antibiotic residues in milk and milk products in different countries. For example, Treiber & Beranek, (2021) found penicillin G and oxytetracycline residues in pasteurized milk and neomycin residues in milk that is raw worldwide. Similarly, Husnain *et al.* (2017) detected penicillin G and amoxicillin residues in raw milk from three towns in Pakistan. In Africa, studies have also reported the presence of various antibiotic residues, including beta-lactam, tetracyclines, aminoglycosides, macrolides, and sulfonamides, in milk samples collected from Tanzania (24% prevalence) and Ethiopia (36% prevalence) (Worku *et al.*, 2017).

Furthermore, Olatoye *et al.* (2016) conducted a study in Nigeria and found antibiotic residues in 40.8% of fresh milk samples, 24.4% of cheese samples, and 62.3% of fermented milk samples. This suggests a higher prevalence of antibiotics throughout the milk value chain, indicating possible misuse of these drugs beyond the farm level. Layada *et al.* (2016) reported that 65.5% of milk samples in Algeria contained antibiotic residues exceeding recommended levels.

In Kenya, the presence of antibiotic residues has also been reported in both raw and pasteurized milk samples. Ahlberg *et al.* (2016) identified beta-lactam, sulfonamides, and tetracyclines in 24% of tested milk samples, while Orwa *et al.* (2017) detected sulphonamides and tetracyclines in raw milk from rural (31.4%) and peri-urban (28.8%) dairy systems. Interestingly, antibiotic residues were found to increase along the milk value chain, with contamination levels rising from the farm (19.5%) to transporters (28.6%) and bulking agents (50%) (Orwa *et al.*, 2017). Kosgey *et al.* (2018) discovered antibiotic residues in 24% of milk samples from vending machines and street vendors in Kenya. Notably, commercial pre-packed milk did not contain any antibiotic residues (Kosgey *et al.*, 2018).

The presence of antibiotic residues in processed and packed milk suggests inefficiency in detecting residues at milk reception and farm levels. This emphasizes the need for improved monitoring and control measures. The accumulation of antibiotic residues in food of animal origin, coupled with the rising prevalence of antibiotic resistance, poses significant risks to human health, agriculture, and the environment on a global scale (Pingault *et al.*, 2017).

However, it is worth noting that the issue of antibiotic resistance in the Kenyan agriculture and dairy sector has not been adequately addressed or investigated. There is a substantial knowledge gap regarding the spread of antibiotic resistance in this sector, which plays a significant role in Kenya's animal-origin food production. Outdated practices, inadequate control over the sales and usage of veterinary medicines, and food safety risks associated with small household farmers are constraints in the Kenyan dairy sector (Kirwa *et al.*, 2021)

Research conducted in the human health sector has shown evidence of antibiotic-resistant *Staphylococcus aureus*, a common source of bovine mastitis. However, there is a lack of research specifically focused on antibiotic resistance in dairy farming. Monitoring results from the National Food Agency in Kenya indicate an increased risk of antibiotic-resistant bacteria development in dairy farming and subsequent spread in the environment through the food chain ((Mbindyo *et al.*, 2021; Sachi *et al.*, 2019).

The global concern of antimicrobial resistance requires a coordinated approach involving multiple sectors. Insufficient legislation and regulation regarding the usage, distribution, import and manufacturing of antimicrobial drugs in many countries contribute to the overuse and misuse of antibiotics. Adopting a One Health approach, which encompasses the environment, animals, plants and humans, is crucial for effective action against antimicrobial resistance (Sachi *et al.*, 2019).

In conclusion, antibiotic residues in animal-derived food products and the emergence of antibiotic resistance pose significant risks to human health and the environment. The prevalence of antibiotic residues in milk and milk products, as well as the increasing presence of antibiotic residues along the milk value chain, highlight the potential misuse and abuse of antibiotics in the agricultural and dairy sectors. In Kenya, there is a lack of research and understanding regarding the spread of antibiotic resistance in the dairy sector, which necessitates further investigation and the adoption of appropriate control measures (Mbindyo *et al.*, 2021).

2.7.2 Processing Challenges in Relation to Antibiotic Residues

The responsibility of preventing antibiotic residues in milk primarily lies with the producer, especially in the case of processed milk and milk products (Van Dijk, 2015). Processors must ensure that the milk they receive is safe and suitable for consumption. The presence of antibiotic residues in milk poses several challenges in the processing of milk and milk products. Dairy starter cultures, including *Lactococcus, Streptococcus, Leuconostoc*, and

Lactobacillus, play a crucial role in the production of various dairy products by producing lactic acid. However, the presence of antibiotic residues interferes with the activity of these starter cultures. For example, in yogurt production, the presence of 60µg/L of penicillin can result in improper yogurt formation (Novés *et al.*, 2015). Studies by Chowdhury *et al.* (2015) have shown that heat processing does not degrade antibiotic residues in cheese and yogurt processing. The antimicrobial resistance of these residues can negatively affect the starter cultures used in the production of fermented milks and cheeses, resulting in poor quality or failed products (Barry, 2017; Gajda *et al.*, 2018; Van Dijk, 2015). Furthermore, the inhibition of starter cultures can have a detrimental impact on product quality (Adetunji & Olatoye, 2012). As noted by Chiesa *et al*. (2020), it's essential to also take into account the repercussions of antibiotic residues on the dairy industry. Antimicrobial compounds can disrupt the manufacturing of dairy products by impeding acid formation, coagulation of milk, and the maturation process of cheeses.

he existence of antibiotics in milk gives rise to additional challenges, including insufficient milk coagulation and flawed maturation of cheeses during the production process, diminished acid and flavor generation in products like buttermilk, restrained growth of starter cultures in reconstituted skim milk, and compromised accuracy of certain quality control tests. These issues can lead to the production of inferior products that may need to be discarded, resulting in significant losses. Moreover, the cleanup of equipment can be costly and time-consuming, leading to disruptions in production schedules (Sachi *et al.*, 2019).

Mastitis infections in cows can further contribute to challenges in milk production. These infections can cause a decline in potassium and lactoferrin levels, which negatively affects the yield of casein, the major protein in milk. Consequently, the cheese production process can be negatively impacted. Additionally, the testing methods for antibiotic residues in milk can be inefficient and complex, making it challenging to accurately evaluate each case and administer appropriate treatment to the animals (Bhosale *et al.*, 2014). In addition, the milk processing industry also incurs losses due to antibiotic-related problems and the reduced chemical and bacterial quality of mastitic milk (Azooz *et al.*, 2020; Mutua *et al.*, 2020).

2.7.3 The Reported Situations of Multiple Drug Resistance Bacteria in East Africa

In recent years, there has been significant research conducted on multiple drug resistance, with a particular focus on antibiotics, in East Africa. Several studies conducted between 2016 and the present have provided insights into the prevalence and patterns of

antibiotic resistance in this region. These findings have highlighted the urgent need to address this growing challenge and implement effective strategies to combat antibiotic resistance (Kikuvi *et al.*, 2016; Mushi *et al.*, 2017; Tesfaye *et al.*, 2019).

A study conducted in Kenya by Kikuvi *et al.* (2016) investigated antibiotic resistance patterns in various sources, including clinical samples, food, and the environment. The research revealed high levels of resistance to commonly used antibiotics such as ampicillin, tetracycline, and sulfamethoxazole. The study emphasized the importance of improved surveillance and infection control measures to tackle the issue of antibiotic resistance in the country.

Similarly, a study conducted in Tanzania by Mushi *et al.* (2017) focused on antibiotic resistance in *Escherichia coli* isolated from different sources. The findings demonstrated elevated levels of resistance to multiple antibiotics, including ampicillin, tetracycline, and ciprofloxacin. This study highlighted the complex dynamics of antibiotic resistance transmission among humans, animals, and the environment in the region.

In Uganda, Ssempijja *et al.* (2018) conducted research on antibiotic resistance patterns in bacteria from poultry farms. The study revealed widespread resistance to multiple antibiotics, such as penicillin, tetracycline, and erythromycin. The findings emphasized the need for better antibiotic stewardship practices within the poultry farming sector to mitigate the spread of antibiotic resistance.

A study conducted in Ethiopia by Tesfaye *et al.* (2019) examined antibiotic resistance in bacteria isolated from clinical samples. The research highlighted the alarming prevalence of multidrug-resistant strains, particularly in Gram-negative bacteria like *Klebsiella pneumoniae* and *Escherichia coli*. The study underscored the urgent need for enhanced infection control measures and antibiotic management strategies in healthcare settings.

Additionally, in Rwanda, Muvunyi *et al.* (2021) conducted research on antibiotic resistance in *Staphylococcus aureus* isolates from clinical specimens. The study revealed high rates of resistance to multiple antibiotics, including methicillin-resistant *Staphylococcus aureus* (MRSA) strains. It emphasized the importance of effective surveillance programs and infection control practices to combat the spread of antibiotic-resistant pathogens in healthcare facilities.

Overall, these studies collectively highlight the significant challenges posed by multiple drug resistance, particularly antibiotic resistance, in East Africa. The findings underscore the urgent need for comprehensive strategies that encompass surveillance, infection control, and antibiotic stewardship to combat the spread of resistant bacteria in the

region. It is crucial to implement multidisciplinary approaches involving healthcare providers, veterinarians, policymakers, and the community to address this pressing public health issue.

Similarly, while antibiotic usage constitutes a significant element of both preventive measures and treatment protocols, the ultimate decision to employ them rests with the farm proprietors, influenced by their own expertise and economic circumstances. Variations in the frequency of antibiotic application can potentially yield disparities in bacterial antibiotic resistance within either the udder or the surrounding environment. Consequently, the aim of this investigation was to ascertain the antibiotic resistance profile of pathogens obtained from quarter milk samples as well as the immediate environment (Dettweiler *et al.*, 2020; Zango *et al.*, 2019).

2.7.4 Withdrawal Time after Drug Administration

The term "withdrawal time" is commonly used to refer to the duration required after administering a drug to a food animal, during which the residue levels of the drug in marketed edible products, such as meats, eggs, and organs, fall below a predetermined maximum residue limit (MRL). The withdrawal time can vary significantly depending on the chemical and physical properties of the drugs and the route of administration (Sachi *et al.*, 2019).

Antimicrobial resistance in milk has become a major concern in recent years. To comply with control policies and ensure effective detection and quantification of antimicrobial resistance in milk, numerous research studies have been conducted worldwide. In the past, some microbiological tests like Delvotest SP-NT and Copan milk tests were commonly used for this purpose (Ndahetuve *et al.*, 2020). However, these tests, although inexpensive, rapid, and easy to perform, lacked sufficient selectivity and accuracy (Oliveri & Anugu, 2021). On the other hand, chromatographic techniques offer higher precision, specificity, and accuracy, but they require proper sample preparation, sophisticated instruments, and well-trained personnel (Sachi *et al.*, 2019).

With the increasing possibility of residues in milk from a wide range of antibiotics, it is a challenge to accurately analyze antimicrobial resistance using a single technique that is cost-effective (Rahman *et al.*, 2021; Widodo *et al.*, 2023). Therefore, there is need to evaluate and compare research studies conducted over the years to identify trends and assess advancements in the analysis of antimicrobial resistance in milk. In addition, there is need to evaluate the various techniques used for determining antimicrobial resistance in milk,

providing insights into the comparative innovation of techniques over time (Kaprou *et al.*, 2021; Michael *et al.*, 2022).

2.8 Food Safety Concerns Related to Antibiotic Residues

Some of the adverse effects observed in humans after consuming milk from cows with mastitis include throat infections, scarlet fever, tuberculosis, and brucellosis. Antibiotic residues, which are frequently found in food products from animal such as milk, possess a an important public health issue, especially when consumed in small quantities over a prolonged period (Song *et al.*, 2015; Van Dijk, 2015). In Tanzania, antimicrobial residues were identified in 36% of milk samples available in the market, implying an average risk of approximately 11 exposures per month for an individual who consumes milk daily (Mdegela *et al*., 2021). Similarly, the risk of consuming milk tainted with antibiotics is substantial in Kenya. This is substantiated by a notable prevalence of antibiotic residues detected at the farm level prior to milk aggregation (Kosgey *et al*., 2018), along the entirety of the value chain (Orwa *et al.*, 2017), and even in ready-to-consume milk and milk products (Wanjala *et al.*, 2018).

According to Nisha (2008), antibiotic residues can have various harmful effects, including the transfer of antibiotic resistance to bacteria that are pathogenic to humans, immune-related pathological effects, autoimmunity, carcinogenicity (e.g., sulphamethazine, oxytetracycline, furazolidone), mutagenicity, nephropathy (gentamicin), hepatotoxicity, reproductive disorders, bone marrow toxicity (chloramphenicol), and allergies in the case of penicillin. Van Dijk (2015) also noted that allergic reactions to certain antibiotics in milk can lead to fatalities in some individuals.

One of the consequences of antibiotic residues in milk is the development of antibiotic resistance. Even low doses of drugs that are insufficient to destroy bacteria have been found to promote the development of resistance as bacteria adapt to survive in such environments. Consequently, when these antibiotics are used to treat infections caused by those pathogens, they prove ineffective (Wanjala *et al.*, 2018). Antibiotic resistance has prompted the World Health Organization (WHO) to compile a list of pathogens that require the development of new antibiotics to effectively control them in humans. Some of these pathogens are commonly found as contaminants in milk and milk products. The increasing prevalence of drug resistance escalates the cost of disease treatment since existing drugs are no longer effective against diseases they once managed successfully. This situation can lead

to fatalities when diseases that were previously manageable become challenging to treat (WHO, 2017).

2.8.1 Antibiotic Residues in Milk

The presence of antibiotic residues in milk can be attributed to various weaknesses within the milk value chain. One of the major risks for contamination of milk with antibiotic residues among farmers is the lack of understanding of the risks associated with antibiotic contamination of food, along with poor or nonexistent treatment records and the absence of a monitoring system. Most antibiotic residues in milk can be traced back to the farmers themselves. For instance, the withdrawal periods for many antibiotics vary, with some requiring up to 7-10 days before milk can be safely consumed after treatment. According to Extension (2007), examples of withdrawal periods for antibiotics in milk include: Penicillin (72 hours), Cloxacillin (48 hours), Pirlimycin (36 hours), Amoxicillin (60 hours), and Cephapirin (96 hours). However, farmers have been known to misuse antibiotics by not adhering to the prescribed doses and withdrawal periods (Kumarswamy *et al.*, 2018). The lack of proper treatment records exacerbates this issue, as farmers may not remember when the animal was last treated. Despite the known dangers of having antimicrobial residues in milk, the misuse of antibiotics continues to persist (Ahlberg *et al.*, 2016; Layada *et al.*, 2016; Olatoye *et al.*, 2016; Worku *et al.*, 2017) resulting in persistent levels of antimicrobial substances in milk and milk products.

Apart from farmers, other stakeholders in the dairy industry may also contribute to the presence of antibiotic residues in milk. Due to inadequate and poor storage facilities for maintaining the cold chain, farmers and other actors in the value chain have resorted to crude methods to enhance the shelf life of milk. One common practice is the addition of antibiotics to raw milk (Ndungu *et al.*, 2016b). Since such practices are illegal, the quantities of antibiotics added may exceed safe levels, and multiple types of antibiotics may be abused. It is crucial to exercise caution at the farm level to ensure that withdrawal periods are strictly observed. This can be achieved through farmer sensitization during training programs and the involvement of qualified veterinary doctors. Additionally, government intervention is necessary, such as controlling the over-the-counter sale of antibiotic drugs to limit their misuse, as well as implementing rigorous market surveillance on milk and milk products.

2.8.2 Effects of Antibiotic Residues on Human Health

Antibiotic residues have been detected in both household and commercially marketed milk in East Africa. When the levels of these residues are sufficiently high, beta-lactams and

oxytetracyclines, commonly used to treat mastitis and respiratory disorders in cattle in the region, can cause hyper-allergic reactions in humans (Brown *et al.*, 2020). Sub-chronic exposure to high residue levels can lead to human health problems such as allergies in susceptible individuals, toxicity, and potential carcinogenic effects, although the veracity of some reactions is sometimes questioned. Individuals with penicillin allergies may develop allergies if they consume milk containing significant amounts of residues from penicillin, as well as other beta-lactam antibiotics like cephalosporins and carbapenems. Tetracycline residues have also been associated with tooth discolouration in young children (Shitandi, 2004b). The widespread use of antibiotics poses a threat to human health as it contributes to the rapid spread of antibiotic resistance. However, a quantitative model to accurately estimate the risks associated with ingested antibiotic residues is yet to be developed. The potential hazards of ingested antibiotic residues alter the human microbiome, promote the emergence and selection of bacterial resistance, and serve as reservoirs for antibiotic resistance in the environment, posing significant concerns for health risks (Ben *et al.*, 2019).

Antibiotic resistance has been observed in various pathogenic bacteria isolated from milk and milk products. A study conducted in the Hawassa area, South Ethiopia, on antibiotic resistance in *Staphylococcus aureus* isolates from cow's milk reported resistance to several antibiotics, including Penicillin G, Ampicillin, Amoxicillin-Clavulanic acid, Ciprofloxacin, Erythromycin, Ceftriaxone, Trimethoprim-Sulfamethoxazole, Oxacillin, and Vancomycin (Daka & Yihdego, 2012). Similarly, a study on lactic acid bacteria isolated from a Spanish goat's milk cheese found that none of the strains were fully susceptible to all tested antibiotics, indicating the presence of multiple resistance (Herreros *et al.*, 2005). These findings have been supported by other researchers who have reported similar antibiotic resistance patterns in milk and milk products (de Almeida Júnior *et al.*, 2015; Wang *et al.*, 2019).

2.9 Uses of Antibiotics (Beta-Lactams) as Enhancers of Healthcare System

Penicillins, widely employed broad-spectrum antibiotics, find frequent usage among a range of medical professionals such as primary care providers, internists, infectious disease specialists, and nurse practitioners. Within the penicillin family, distinct subcategories exhibit differences in terms of pharmacokinetics, coverage of bacterial strains, safety profiles, and cost implications. These variations furnish clinicians with a spectrum of options to consider when determining the appropriate medication for a particular situation (Shaker & Shaaban, 2017; Sotgiu *et al.*, 2016).

The use of beta-lactams requires coordination among an inter-professional animal healthcare team. Veterinarians are responsible for ordering or prescribing the antibiotics, and administering them. They engage in several critical tasks involving penicillins, including medication reconciliation, which involves reviewing a ill animals' medication history for accuracy and identifying potential discrepancies. They also assess potential drug interactions to ensure the safety and efficacy of treatment. Furthermore, they reinforce proper administration instructions to farmers for their animals. Additionally, they continuously evaluate the therapeutic effectiveness of the prescribed antibiotics to monitor the progress of treatment. (Shaker & Shaaban, 2017). In addition, they verify the dosing and duration of therapy and communicate with the prescriber if any discrepancies are encountered. Comprehensive awareness regarding the potential occurrence of anaphylactic reactions linked to beta-lactam agents and the potential for crossover allergies is crucial for every member of the animal healthcare team. Effective communication of this information within the team when required is essential. Despite the prevalent use of beta-lactams, their proper prescription necessitates a collaborative approach among various healthcare professionals, ensuring the attainment of optimal outcomes (Sotgiu *et al.*, 2016; Vrancianu *et al.*, 2020).

2.10 Beta-Lactam Antibiotics, Types and Mechanisms of Action

Beta-lactam antibiotics have revolutionized the treatment of bacterial infectious diseases since their discovery in the 1930s. They are one of the most commonly prescribed drug classes and account for a significant portion of the antibiotics market, with an estimated annual expenditure of $15 billion USD, making up 65% of the total antibiotics market (Pandey & Cascella, 2019).

From a biochemical standpoint, beta-lactam antibiotics exhibit a shared characteristic: the presence of a reactive 3-carbon and 1-nitrogen ring, recognized as the beta-lactam ring. These antibiotics can be categorized into distinct types. The initial category is penicillins, encompassing natural penicillins, beta-lactamase-resistant agents, aminopenicillins, carboxypenicillins, and ureidopenicillins. Penicillins possess a nucleus of 6-aminopenicillanic acid, along with diverse side chains (Ferri *et al.*, 2017; Gorssen *et al.*, 2021; Pandey & Cascella, 2019).

The second type is the cephalosporins, which contain a 7-aminocephalosporanic acid nucleus and side chains with 3,6-dihydro-2H-1,3-thiazane rings. Cephalosporins are traditionally divided into five classes or generations, although the terminology may vary (Jyoti & Amandeep, 2020). Carbapenems form the third type of beta-lactam antibiotics and

are reserved for the treatment of severe bacterial infections, especially those caused by multidrug-resistant pathogens. They have a carbapenem coupled to a beta-lactam ring, providing protection against most beta-lactamases. However, resistance to carbapenems has become a significant concern, particularly among gram-negative bacteria such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Hawkey & Livermore, 2012).

Monobactams constitute another type of beta-lactam antibiotics. They differ from other beta-lactams as their beta-lactam ring is not fused to another ring. Monobactams are primarily effective against aerobic gram-negative bacteria and are considered inactive against gram-positive bacteria (Torres & Blanca, 2010). They inhibit bacterial cell wall synthesis and are bactericidal. The development of resistance to beta-lactam antibiotics is a major global health issue. Resistance mechanisms include genetic mutations, the production of beta-lactamases, decreased penetration to the target site, efflux pumping, and alteration of target site PBPs (Letourneau & Calderwood, 2019; Pandey & Cascella, 2019). Overcoming resistance and ensuring the efficacy of beta-lactam antibiotics require ongoing research and development of new antibiotics with enhanced spectrum efficiency against resistant bacteria (Lima *et al.*, 2020; Sachi *et al.*, 2019).

Beta-lactamase inhibitors, such as clavulanic acid, sulbactam, tazobactam, avibactam, and vaborbactam, work by inactivating serine beta-lactamases, enzymes that hydrolyze and inactivate the beta-lactam ring. Some of these inhibitors are active against carbapenemases, such as *Klebsiella pneumoniae* carbapenemase (Jyoti & Amandeep, 2020; Pandey & Cascella, 2019). The efficacy of beta-lactam antibiotics is attributed to their ability to inhibit the synthesis of the peptidoglycan layer, a vital component of bacterial cell walls. They irreversibly bind to penicillin binding proteins (PBPs), preventing the final crosslinking of the nascent peptidoglycan layer and disrupting cell wall synthesis. The structural similarity between beta-lactam antibiotics and d-alanyl-d-alanine, a component of the peptidoglycan layer, facilitates their binding to PBPs (Catherwood *et al.*, 2020; Sauvage & Terrak, 2016).

The misuse and overuse of antibiotics, including beta-lactams, contribute to the emergence and spread of antimicrobial resistance. This resistance poses a significant challenge for public health, as it affects the efficacy of these vital drugs. Strategies to combat antimicrobial resistance include appropriate antibiotic stewardship, infection control measures, and the development of new antibiotics (Ferri *et al.*, 2017; Pandey & Cascella, 2019).

In livestock production systems, antibiotic usage is a significant driver of antimicrobial resistance. Livestock farming creates favorable conditions for bacteria to acquire resistance genes and facilitates the transmission of resistant bacteria to humans and the environment. Monitoring antibiotic usage in livestock is crucial for understanding its impact and implementing appropriate measures. Detection of antibiotic residues in animal products, such as meat and milk, is also essential to ensure consumer safety and prevent the presence of antibiotics above permissible limits (Sharma *et al.*, 2018).

In conclusion, beta-lactam antibiotics have played a vital role in combating bacterial infections. However, the emergence of resistance poses a significant challenge. Ongoing research is focused on developing new antibiotics and improving detection methods to ensure their efficacy, while measures are needed to regulate and monitor antibiotic usage in livestock to mitigate the risk of antimicrobial resistance.

2.10.1 Beta-Lactam Antibiotics Usage on Different Microorganism

The applications of beta-lactam antibiotics are diverse and differ based on the specific subclass being considered (King *et al.*, 2017). Natural penicillins, such as penicillin G (administered intravenously) and penicillin V (administered orally), are derived from natural sources and are used to treat selected infections caused by both gram-positive and gram-negative bacteria. These antibiotics have shown efficacy against a range of conditions, including *Streptococcus pneumoniae* and *meningitis*, *Streptococcal pharyngitis*, endocarditis, skin and soft tissue infections, *Neisseria meningitidis* infections, and syphilis (King *et al.*, 2017; Pandey & Cascella, 2019). Although penicillin antibiotics have been widely utilized, ongoing research is focused on enhancing their effectiveness against various gram-positive microorganisms.

2.10.2 Beta-Lactamase Enzyme Resistant Agents

The agent oxacillin (administered intravenously), nafcillin (administered intravenously), and dicloxacillin (administered orally) are effective against gram-positive organisms. Despite the prevalence of resistance among *Staphylococci*, these antibiotics remain the preferred choice for managing methicillin-susceptible *Staphylococcus aureus* (MSSA) infections, including skin and soft tissue infections, as well as serious MSSA infections. The development of resistance in this species is a result of continued exposure, leading to the phenomenon of resistance or adaptation by microorganisms (King *et al.*, 2017; Letourneau & Calderwood, 2019; Pandey & Cascella, 2019).

Amino-penicillin antibiotics exhibit activity against both gram-positive and gram-negative bacteria, including many *Enterobacteriaceae* and anaerobic organisms. They are often used in combination with beta-lactamase inhibitors. Amino-penicillins, akin to penicillin in chemical structure, exhibit a wider range of activity. Unlike penicillin, they are not rendered inactive by acid hydrolysis, facilitating oral administration. However, they are vulnerable to degradation by beta-lactamase enzymes and are consequently sometimes used alongside beta-lactamase inhibitors. Amino-penicillins are effective against a majority of gram-positive bacterial infections and also demonstrate efficacy against certain gram-negative infections such as *Escherichia coli* and *Haemophilus influenzae*. They are used in the treatment of upper and lower respiratory tract infections, endocarditis, urinary tract infections, skin infections, and others (King *et al.*, 2017; Vrancianu *et al.*, 2020).

Amoxicillin (administered orally) and ampicillin (administered orally or intravenously) are primarily used for controlling upper respiratory tract infections (such as sinusitis, pharyngitis, and otitis media), *Enterococcus faecalis* infections, and *Listeria* infections. They are also active against microbes that produce beta-lactamase, and can be combined with beta-lactamase inhibitors, such as amoxicillin/clavulanate (administered orally) and ampicillin-sulbactam (administered intravenously). Additionally, they are effective in the treatment of upper respiratory tract infections (sinusitis, otitis media), intra-abdominal infections, and complicated urinary tract infections (Vrancianu *et al.*, 2020; Yahav *et al.*, 2020).

The ureidopenicillin piperacillin is active against aminopenicillin-resistant gram-negative *bacilli*, specifically *Pseudomonas aeruginosa*. It is commonly used in combination with beta-lactamase inhibitors. It is important to note that these drugs work well in combination with two or more antibiotics. All ureidopenicillins have similar spectra of activity and pharmacological properties. Azlocillin and mezlocillin are no longer available in clinical practice, as they have been replaced by piperacillin-tazobactam (Saxena *et al.*, 2019).

Piperacillin displays activity akin to ampicillin against gram-positive species. It demonstrates remarkable efficacy against Streptococcal species, Neisseria, Haemophilus, and several members of the Enterobacteriaceae family. Additionally, it exhibits robust activity against both cocci and bacilli belonging to anaerobic species. Piperacillin has the capacity to inhibit 60% to 90% of Pseudomonas aeruginosa strains at concentrations lower than 16 µg/mL. Analogous to ampicillin, it is susceptible to hydrolysis by class A beta-lactamases (Saxena *et al.*, 2019; Vrancianu *et al.*, 2020; Yahav *et al.*, 2020).

Cephalosporins pertain to a category of beta-lactam antibiotics. The first generation of cephalosporins encompasses cefazolin (administered intravenously), cephalexin (administered orally), and cefadroxil (administered orally). They prove effective in managing skin and soft tissue infections, significant Methicillin-Susceptible Staphylococcus Aureus (MSSA) infections, and as prophylactic measures during perioperative surgeries. Certain first-generation cephalosporins are employed as preventive antibiotics for surgical procedures involving the pelvis, abdomen and chest (Mehta & Sharma, 2016).

Second-generation cephalosporins include cefuroxime (administered intravenously or orally), cefoxitin (administered intravenously), cefotetan (administered intravenously), cefaclor (administered orally), and cefprozil (administered orally). They are used to treat upper respiratory tract infections (sinusitis, otitis media), gynecologic infections (cefotetan, cefoxitin), and perioperative surgical prophylaxis. They may also be used for ear infections, sinus infections, urinary tract infections, gonorrhea, meningitis, and sepsis (Gallagher & MacDougall, 2016; Pandey & Cascella, 2019).

Third-generation cephalosporins include cefotaxime (administered intravenously), ceftriaxone (administered intravenously), cefpodoxime (administered orally), cefixime (administered orally), cefdinir (administered orally), cefditoren (administered orally), and ceftibuten (administered orally). They are used for the treatment of community-acquired pneumonia, meningitis, urinary tract infections, *streptococcal* endocarditis, *gonorrhea*, and severe Lyme disease. Fourth-generation cephalosporins include anti-pseudomonal cephalosporins such as ceftazidime (administered intravenously), ceftazidime/avibactam (administered intravenously), and cefepime (administered intravenously). Fifth-generation cephalosporins include ceftolozane/tazobactam (administered intravenously). These antibiotics have various uses, including penetration into the cerebrospinal fluid for the treatment of meningitis and nosocomial infections like pneumonia. Ceftazolone plus a beta-lactamase inhibitor is used for the treatment of complicated urinary tract infections (Saxena *et al.*, 2019; Yahav *et al.*, 2020).

Cephalosporins such as ceftaroline (administered intravenously) and ceftobiprole (administered intravenously), which are also classified as fifth-generation cephalosporins, are used to treat community-acquired pneumonia, hospital-acquired *pneumonia* (excluding ventilator-acquired pneumonia), and skin and soft tissue infections. They can also be used for the treatment of gonorrhea, pneumonia, strep throat, urinary tract infections, and they penetrate rapidly into the tissues, making them suitable for serious infections, including meningitis (Yahav *et al.*, 2020). Carbapenems, including imipenem/cilastatin (administered

intravenously), meropenem (administered intravenously), and doripenem (administered intravenously), are used for nosocom.

2.10.3 Mechanism of Beta-Lactam Action and the Associated Adverse Effects

To comprehensively grasp the impact of beta-lactamases on the efficacy of beta-lactam antibiotics within our therapeutic arsenal, it is imperative to briefly revisit the mechanism through which beta-lactams counter bacterial infections. The bactericidal influence of beta-lactam antibiotics is exerted by obstructing penicillin-binding proteins (PBPs), enzymes integral to the synthesis of the bacterial cell wall (Sotgiu *et al.*, 2016). The structural integrity of the bacterial cell wall plays a pivotal role in upholding cellular shape in challenging environments such as serum, urine, lung mucus, or the gastrointestinal tract, which can be hypertonic and inhospitable. This cell wall comprises alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) units that are interconnected by transglycosidases. Each NAM unit is linked to a pentapeptide, and the PBPs function as transpeptidases, catalyzing the linkage of two d-alanine-d-alanine NAM pentapeptides. This linkage provides the requisite rigidity for the cell wall. In the 1960s, Strominger noted the resemblance between the beta-lactam ring's structure and that of the d-alanine-d-alanine within the NAM pentapeptide (Drawz & Bonomo, 2010; Fisher & Mobashery, 2014; Fishovitz *et al.*, 2015). Subsequently, the penicillin-binding proteins (PBPs) mistakenly incorporate the beta-lactam structure as a constituent during the process of cell wall synthesis. This prompts acylation of the PBP, rendering the enzyme inept at facilitating subsequent transpeptidation reactions. Consequently, the synthesis of the cell wall decelerates, while the constant peptidoglycan autolysis, steered by bacterial autolytic enzymes referred to as amidases, persists. The deterioration of the murein sacculus, the peptidoglycan lattice that envelops the bacterium, undermines the integrity of the cell wall and heightens its permeability. As a result, the curtailment of transpeptidation through beta-lactams culminates in cell lysis (Bonomo, 2017).

Compared to other antibiotic classes, beta-lactam agents are generally safe and well-tolerated (Pandey & Cascella, 2019). The most common side effects are allergic reactions, which occur in a range of 0.7% to 10% of cases. These reactions typically manifest as maculopapular rashes, although reports of anaphylaxis occur in 0.004% to 0.015% of patients. Apart from allergic reactions, beta-lactams can also induce organ-specific adverse effects (Torres *et al.*, 2019).

Penicillin G and piperacillin have been linked to impaired hemostasis due to compromised platelet aggregation. The intravenous administration of benzathine penicillin G has exhibited a correlation with instances of cardiorespiratory arrest and mortality. Cephalosporins have been sporadically associated with bone marrow depression, including cases of granulocytopenia. Certain cephalosporins possess the potential to be nephrotoxic and can lead to renal tubular necrosis. Ceftriaxone, by displacing bilirubin from albumin, can result in neonatal jaundice and may induce biliary pseudolithiasis due to its heightened affinity for biliary calcium. At elevated doses or in patients with renal dysfunction, cefepime has been associated with encephalopathy and nonconvulsive status epilepticus. Imipenem, when administered in high dosages to patients with CNS lesions or renal insufficiency, has been linked to the occurrence of seizures (Bonomo, 2017; Torres *et al.*, 2019).

It is important to note that although these adverse effects can occur, they are generally infrequent, and the benefits of beta-lactam antibiotics in treating bacterial infections often outweigh the potential risks.

2.10.4 Beta Lactam Antibiotics and Beta-Lactamase Enzyme

In a study conducted in Kenya, Orwa *et al.* (2017) found the presence of tetracyclines and sulfonamides in rural and peri-urban dairy systems, while Ahlberg *et al.* (2016) reported the presence of beta-lactam antibiotics, tetracyclines, and sulfonamides in milk, with beta-lactams being more prevalent than tetracyclines and sulfonamides. Ahlberg *et al.* (2016) reported detection rates of 5% for beta-lactams, 2.5% for sulfonamides, and 0.6% for tetracyclines. In the Makueni area, the rates of detection were 53.3%, 26.7%, and 6.7% for beta-lactams, sulfonamides, and tetracyclines, respectively. Beta-lactams, especially penicillin, are commonly used antibiotics in the management of bovine mastitis caused by *E. coli* and *A. pyogenes (*Sharun *et al.*, 2021). As a result, beta-lactam antibiotic residues are frequently reported in milk (Sachi *et al.*, 2019).

Beta-lactam antibiotics, including penicillin, monobactams, carbapenems, and cephalosporins, possess a beta-lactam ring structure that gives them their antibacterial activity. All beta-lactam antibiotics share a common structural element, a four-atom ring called a beta-lactam ring. These antibiotics can have a four-membered beta-lactam ring, a six-membered ring (found in penicillins, carbapenems, and monobactams), or a seven-membered ring (found in cephamycins and cephalosporins) (Pandey & Cascella, 2020). The mode of action of beta-lactam antibiotics involves inhibiting the synthesis of bacterial cell walls, leading to cell lysis and death. They achieve this by binding and acylating the active site of

penicillin-binding proteins (PBPs), which are enzymes essential for bacterial cell wall biosynthesis. The four-membered beta-lactam ring in their structure plays a critical role in their mechanism of action (Pandey & Cascella, 2020). Beta-lactam antibiotics target a group of membrane-anchored enzymes in bacteria involved in crosslinking the bacterial cell wall. By binding to these enzymes, beta-lactams prevent them from carrying out their essential function, resulting in cell death due to osmotic instability (Bush & Bradford, 2016). This property makes beta-lactam antibiotics valuable in human medicine (King *et al.*, 2017).

Bacterial resistance to beta-lactam antibiotics can occur through the production of beta-lactamase enzymes, which can degrade beta-lactam antibiotics. Beta-lactamase (BLs) enzymes, also known as penicillinases, were first identified in *E. coli* and have been found in various bacteria. Currently, there are over 800 different beta-lactamases, each with specific targets among different organisms and drugs (Bush & Bradford, 2020). Both gram-positive and gram-negative bacteria produce these enzymes, and most of them act against penicillins or cephalosporins (Munita & Arias, 2016). Beta-lactamases specifically hydrolyze the beta-lactam ring present in antibiotics such as penicillin, cephalosporins, monobactams, and carbapenems, conferring resistance against these antibiotics. However, it has been reported that they show poor hydrolysis activity against cephalosporins, carbapenems, or monobactams compared to benzyl penicillin. Different beta-lactam antibiotics have varying susceptibility to beta-lactamase activity, with seven-membered cephalosporins being more resistant (Pandey & Cascella, 2022).

Various methods have been explored to manage antibiotic residues in milk, including the use of enzymes to degrade specific antibiotic residues. For example, beta-lactamase enzymes have been used to break down beta-lactam residues into other compounds that are microbiologically ineffective. Horton *et al.* (2015) reported that the use of microorganisms producing cefotaximase was the most effective method for reducing cefquinome levels in milk. In the medical industry, beta-lactamase enzymes have been used to remove antibiotic residues from medical instruments to prevent allergic reactions in patients (Bush and Jacoby, 2010). The use of beta-lactamase to degrade penicillin G, penicillin V, and ampicillin in milk has been reported. However, the safety of products made from such milk is not guaranteed, and the use of beta-lactamase as a food additive is not recommended (Wang *et al.*, 2013; Zhou *et al.*, 2015).

2.10.5 Contraindications and Monitoring of Antibiotics

Individuals with a history of anaphylactic reactions or severe skin conditions like Stevens-Johnson syndrome and toxic epidermal necrosis should steer clear of using penicillin antibiotics (Bonomo, 2017; Torres *et al*., 2019). However, penicillins are deemed safe for usage during pregnancy and while breastfeeding, as the drug is present in breast milk at low concentrations. In instances of renal impairment, adjustments to penicillin doses are necessary, particularly in cases of end-stage renal disease. For such patients, an initial full loading dose is administered, followed by half the loading dose every 8 to 10 hours or 4 to 5 hours, contingent on the glomerular filtration rate (Saxena *et al.*, 2019).

The majority of penicillins exhibit a brief half-life, typically spanning less than an hour. In cases of parenteral administration, these agents are commonly administered every four hours, particularly when addressing severe systemic infections in patients with unimpaired renal function. However, dose adjustments are necessary for certain penicillins like piperacillin and ampicillin when administered to patients with renal insufficiency. On the other hand, penicillin agents such as nafcillin, oxacillin, cloxacillin, and dicloxacillin are excreted primarily through the liver and bile, requiring no modification in the case of renal impairment (Mohiuddin *et al.*, 2019). Most penicillins reach therapeutic levels in fluids such as pleural, pericardial, peritoneal, synovial fluids, and urine. However, penetration into the cerebrospinal fluid (CSF) is limited in the absence of inflammation, but therapeutic levels can be achieved in cases of meningitis (Kovalenko, 2019).

2.11 Screening Methodologies and Cost Related

Confirmatory analysis procedures and methodologies can be costly in terms of time, equipment, and chemicals. They also require highly trained personnel with expertise in the field. Control laboratories often have to analyze a large number of samples with various analytes within short timeframes. Therefore, there is a need for screening methods that can efficiently analyze a large number of samples in a short period. These methods should have high throughput and low cost. Screening methodologies need to possess the capability to identify analytes or analyte classes at the intended level of significance (Stokes et al., 2020; Toldra & Reig, 2006). While some cases of false positives (misidentified as compliant) might be acceptable since they can be subjected to additional confirmatory analysis, it is essential for the screening approach to minimize the occurrence of false negative outcomes (wrongly identified as non-compliant). False negatives should be minimized as these instances would

not undergo subsequent examination. The subsequent requirements are typically taken into account for a screening method.

Effective preparation procedures for sampling, particularly for solid foods commonly found in animal-origin products, are paramount for boosting the sensitivity of screening tests. Diverse extraction techniques, often grounded in solid-phase extraction, are employed for sample purification. The selection of cartridges for use depends on the specific analytes and aims to eliminate potential interferences that might be present in food samples (Bylda *et al.*, 2014). The limits of detection in screening methods typically hinge on the preparatory extraction and purification processes applied to the sample.

Immunological methods, such as ELISA test kits, are commonly used for screening residues in animal foods. There are numerous commercially available kits for this purpose. Other immunological methods, such as radio-immunoassay, and more recently, biosensor-based methods, have also become available. Chromatographic methods primarily involve High Performance Thin-Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC), coupled with different detection systems (Sohrab *et al.,* 2021).

2.11.1 Methods of Detecting of Beta-Lactams Residues

There are two primary methods employed in the detection of antibiotics in food animals: qualitative and quantitative tests. Qualitative tests involve determining the presence or absence of specific chemicals in a sample, and methods such as thin layer chromatography and rapid test kits are commonly used (Rebe Raz *et al.*, 2009). These methods are limited to identifying the residue components in food. On the other hand, quantitative tests aim to determine the absolute or relative abundance, often expressed as a concentration, of specific substances in a sample. Microbiological and immunological methods are utilized for the detection of antimicrobial residues in milk and muscle.

Effective monitoring of the presence of antimicrobial drug residues in food heavily hinges on the availability of appropriate analytical techniques (Rebe Raz *et al*., 2009). In the context of milk, beta-lactam antibiotics are generally screened using nonspecific methods. While these methods are quick and sensitive, they lack quantitativeness and can sometimes produce false positive outcomes (Holstege *et al*., 2002). Presently, there exists a demand for a high-throughput screening method that offers a wide-ranging detection scope (Rebe Raz *et al.*, 2009). Various methods have been employed for the detection and analysis of antibiotic residues in milk. For instance, Nisha (2008) mentions Enzyme-Linked Immunosorbent Assay (ELISA), HPLC, liquid chromatography, gas chromatography, and paper chromatography as means of analyzing drug residues. Chromatographic methods have been commonly used by

researchers for the detection and quantification of antibiotic residues in milk. Tang *et al.* (2009) utilized high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry to simultaneously determine five fluoroquinolone residues in milk. Chung *et al.* (2009) employed HPLC and microbial assays to detect and analyze sulfonamides and quinolones in cow's and goat's milk, while Ramirez *et al.* (2003) developed a method using high-performance thin-layer chromatography (HPTLC) combined with bioautography to identify and quantify chloramphenicol, ampicillin, benzyl penicillin, dicloxacillin, and erythromycin residues in cow's milk. Kaya and Filanzi (2010) used thin-layer chromatography and bioautographic methods to detect penicillin G, oxytetracycline, gentamicin, streptomycin, and neomycin, while Khaskheli *et al.* (2008) employed HPLC to detect beta-lactam residues. Aguilera-Luiz *et al.* (2008) used ultra-high-pressure liquid chromatography coupled with tandem quadrupole mass spectrometry to determine quinolones, sulphonamides, macrolides, anthelmintics, and one tetracycline.

In addition to chromatographic methods, other techniques have also been used. For example, Rebe Raz *et al.* (2009) developed a microarray biosensor based on an imaging surface plasmon resonance platform for the quantitative and simultaneous immune detection of different antibiotic residues in milk. Vera-Candioti *et al.* (2010) utilized capillary zone electrophoresis coupled with diode array detection for the analysis of beta-lactam, tetracycline, quinolone, amphenicol, and sulfonamide residues. Song *et al.* (2015) combined a multi-colour quantum dot-based immunofluorescence assay with array analysis to achieve simultaneous, sensitive, and visual detection of streptomycin, tetracycline, and penicillin G in milk.

Inhibition of microorganisms has also been utilized as a testing method. For example, Khaskheli *et al.* (2008) and Gaudin *et al.* (2004) employed the five-plate test, known as the Screening Test for Antibiotic Residues (STAR), to screen for various groups of antibiotics. However, these tests are reported to be less practical in a farm setting due to their complexity, resource requirements, and time-consuming nature (Ahlberg *et al.*, 2016).

Detection and quantification kits based on some of these methods have also been used. Yamaki *et al.* (2004) utilized the Delvo test SP kit for the detection of beta-lactam and sulfonamide residues. Kurwijila *et al.* (2006) used the Charm-AIM screening test kit for the detection of beta-lactam, tetracycline, aminoglycosides, macrolides, and sulfonamides, and Ahlberg *et al.* (2016) employed the Delvo test screening test and the Trisensor test for the detection of beta-lactam, sulfonamide, and tetracycline residues. However, despite their usefulness, these tests and developed methods may have limitations. For instance, Ahlberg *et*

al. (2016) found conflicting results between the Delvo test and HPLC analysis, indicating the presence of antibiotics in milk according to the Delvo test but not confirmed by HPLC. In another study by Layada *et al.* (2016), a microbial inhibition assay, the Delvo test SP-NT test kit, and liquid chromatography coupled to mass spectrometry (LC-MS/MS) were used.

To ensure food safety and minimize processing losses, testing for residues in raw milk should be a parameter for milk acceptance. However, implementing ideal methods at the farm level is challenging, and the various rapid methods available sometimes yield conflicting results. It is crucial to develop a reliable and fast method applicable both at the farm and processor levels. Sensitizing farmers about observing withdrawal periods and enhancing consumer awareness regarding food safety are also important. Appendix 3 provides an overview of the different testing methods applied in the detection of antibiotic residues in milk.

2.11.2 Techniques for Detection of Residues

Various techniques have been employed for the detection of antibiotic residues (Ars) in milk, including chromatographic, immunological, microbiological, and miscellaneous methods. Among these, chromatographic techniques have been the most widely used (51.34%), followed by immunological methods (25.89%), microbiological methods (16.96%), and miscellaneous techniques (8.04%) (Gaudin, 2017).

Chromatographic techniques have gained popularity, especially in recent times, due to their higher sensitivity, specificity, and quantification capabilities. They offer advantages over other methods, making them the preferred choice for detection. On the other hand, immunological and microbiological techniques can be more cost-effective and rapid but are generally less efficient, and their quantification and detection capabilities may not be as satisfactory (Gaudin, 2017).

2.11.3 Determination and Detection Antibiotics Residues and their stability in cow's milk

Detecting and quantifying antibiotic residues in cow's milk are pivotal stages in upholding the safety and excellence of dairy products. An array of analytical methods has been devised to accomplish precise detection, spanning from conventional techniques like high-performance liquid chromatography (HPLC) and gas chromatography (GC), to more cutting-edge approaches such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assays (ELISA). These methods enable the identification and quantification of specific antibiotics or their metabolites in milk samples,

allowing for effective monitoring of residue levels. To enhance sensitivity and selectivity, sample preparation techniques like solid-phase extraction (SPE) and matrix solid-phase dispersion (MSPD) are often employed to remove interfering compounds and concentrate the target analytes. The development of biosensors, utilizing aptamers, antibodies, or other recognition elements, has also gained traction due to their potential for real-time and on-site analysis (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020).

Ensuring the stability of antibiotic residues in cow's milk throughout processing, storage, and distribution is equally vital. Factors such as temperature, pH, and light exposure can influence the degradation kinetics of these residues. In some cases, the breakdown of antibiotics can result in the formation of degradation products, some of which may also be harmful. Therefore, studies on the stability of antibiotic residues under different storage conditions are essential to understand the potential risks associated with the consumption of milk containing these residues. Moreover, the development of rapid stability assessment methods can aid in predicting the shelf life of milk products and inform proper storage practices. Integrating such stability studies with comprehensive residue monitoring approaches contributes to safeguarding the integrity of dairy products and minimizing potential health hazards posed by antibiotic residues (Jyoti & Amandeep, 2020).

2.11.4 Thermal kinetics of Antibiotics (azithromycin and tetracycline)

The stability of antibiotic residues in cow's milk is a critical aspect with significant implications for food safety. Numerous studies have delved into the effects of temperature on the stability of antibiotics in milk, investigating degradation rates and potential reduction in antimicrobial activity due to thermal treatment. However, the outcomes of these studies exhibit variability based on factors such as treatment temperature, solvent used, and pH conditions. Specifically, research focused on the thermal kinetics of antibiotics like azithromycin and tetracycline has revealed degradation rate constants (k) that shed light on their stability profiles (Imperial & Ibana, 2016; Jyoti & Amandeep, 2020). For instance, the degradation rate constants (k) of azithromycin have been studied across various temperatures. At 4°C, the k value was found to be 1000 ± 8.10^{-4} after 1 hour, plummeting to 62 ± 2.10^{-4} after 24 hours. Conversely, at 37°C, the k value increased from 50 ± 1.10^{-4} to 966 ± 9.10^{-4} after 3 hours of incubation and gradually decreased thereafter. Further, at 70°C, a significant increase in k was observed after 3 hours (4033 ± 22.10^{-4}), which decreased to 537 ± 5.10^{-4} after 24 hours of incubation. The stability trend was also observed at 100°C, where the k value initially increased from 200 ± 3.10^{-4} to 3900 ± 18.10^{-4} after 3 hours and then decreased

gradually (Imperial & Ibana, 2016). Similarly, the stability of tetracycline was evaluated across temperatures. At 4°C, the k value was 20 ± 4.10^{-4} after 1 hour, notably increasing to 256 ± 32.10^{-4} after 3 hours and gradually decreasing to 50 ± 8.10^{-4} after 24 hours. At 37°C, the k value surged from 50 ± 6.10^{-4} to 400 ± 46.10^{-4} after 3 hours and then decreased to 135 ± 20.10^{-4} after 24 hours. Interestingly, the k value experienced substantial elevation at 70 and 100°C (from 30 ± 6.10^{-4} to 630 ± 81.10^{-4} and from 60 ± 15^{-4} to 860 ± 61^{-4}, respectively) after 3 hours of incubation, followed by a subsequent decrease.

The results highlight that the stability of these antibiotics, like azithromycin and tetracycline, is intricately linked to temperature variations. Notably, both antibiotics exhibited relatively stable profiles at 4°C and 37°C over a 24-hour timeframe. This stability aligns with their good antibacterial activities, as evidenced by their zones of inhibition against bacteria like *B. subtilis*. However, azithromycin's antibacterial activity diminished after exposure to 70 and 100°C, indicating a loss of effectiveness (Imperial & Ibana, 2016).

2.11.5 pH Effect on the stability of azithromycin and tetracycline

The study by Konaklieva (2014) delved into the impact of varying pH levels on azithromycin's stability, yielding significant insights. An interesting pattern emerged, highlighting a substantial increase in the degradation rate constant (k) of azithromycin at acidic pH levels of 4 and 5 after 1 hour of incubation. This increase was followed by a noteworthy decrease after 24 hours. In contrast, azithromycin's constant k values were minimal at pH 8 and 9. Similar pH-driven effects were also observed for tetracycline's stability, with distinct trends across different time points, consistently pointing to an increase in constant k values with decreasing pH levels. These trends persisted across different time points, consistently indicating an increase in constant k values with decreasing pH levels (Imperial & Ibana, 2016; Konaklieva, 2014).

The study's findings closely align with previous research, underlining the pivotal role of pH in antibiotic stability. Various antibiotics, including penicillin G, tetracycline, and oxytetracycline, have exhibited differential stability profiles in response to varying pH levels. For instance, a decrease in pH from 11 to 3 resulted in reduced penicillin G stability, with alkaline pH proving more conducive for stability. Similarly, tetracycline's stability and degradation patterns in food products were notably influenced by pH variations. These observations highlight that the degradation of antibiotics is influenced not only by temperature but also by the pH of the environment (Konaklieva, 2014).

In conclusion, the study revealed that raw milk samples contained concentrations of azithromycin and tetracycline residues exceeding FDA-prescribed limits, effectively inhibiting the growth of B. subtilis. Elevated temperatures of 70 and 100°C notably impacted the stability and antibacterial activity of azithromycin, while tetracycline's complete elimination wasn't achieved at the same temperatures. Furthermore, the study showcased how acidic pH levels significantly reduced the stability of both azithromycin and tetracycline, in contrast to alkaline pH conditions. These findings indicate an urgent public health concern due to the elevated antibiotic residue levels in milk samples, necessitating immediate action to ensure the safety of dairy products consumed by the public.

The study's implications are profound, calling for stringent screening of milk for antibiotic residues before reaching consumers. This practice can significantly mitigate the risk of residual contamination in the food chain and safeguard consumer health. Overall, the study underscores the importance of understanding the multifaceted factors influencing antibiotic stability in food products, encompassing temperature, pH, and adherence to safety guidelines.

2.11.6 Microbiological Approaches in Antibiotic Detection

Microbial inhibition assays are widely employed to identify antibiotic residues in milk. These procedures encompass placing a susceptible microorganism in the milk sample for incubation. In cases where no antibiotics are present, the microorganism thrives, evident through changes like agar medium opacity or color shifts attributed to acid formation. Conversely, when antibiotics or inhibitors are present, the microorganism fails to thrive, leading to the development of a zone of inhibition or absence of color change (Gaudin *et al.*, 2018).

The *Bacillus stearothermophilus* disc diffusion assay (BsDA) is a widely used microbial inhibition test for monitoring growth inhibition. It is an official standard test in reference laboratories for regulatory use. In this method, a paper disk soaked in the sample to be tested is placed on the surface of an agar medium containing *B. stearothermophilus var. calidolactis*. Incubation leads to opacity of the agar medium in the presence of the test strain, while zones clear of the strain are formed if the sample contains antibiotic residues (Sachi *et al.*, 2019; Torres *et al.*, 2019).

The Delvo test, developed by Gist-brocades BV, is another well-known microbial inhibitor test. The Delvo test P, designed to detect beta-lactams, uses *B. stearothermophilus* encapsulated in an agar medium with a pH indicator and a nutrient tablet. The milk sample is

dispensed onto the agar surface. Incubation at 64°C for 2½ hours results in a colour change from purple to yellow if the test is negative. The Delvo test SP is capable of detecting a wider range of substances and requires incubation for 23-24 hours. These tests have been used worldwide and have high sensitivities for detecting specific antibiotics (Fishovitz *et al.*, 2015).

Other microbial inhibition tests include the Charm AIM-96 test, Charm Farm Test-OvialO, Charm Farm Test-Omini VialO, Brilliant Black Reduction Test, Eclipse 100, STAR five-plate test, and Copan microbial inhibitor test. These tests are designed to detect various antibiotics and employ liquid mediums instead of agar. Incubation times vary, and the presence or absence of antibiotic residues is indicated by colour changes or other visual observations (Jevtusevskaja *et al.*, 2016; Kantiani *et al.*, 2009).

In recent years, new microbial inhibition assays, such as the Delvo test SP-NT (sulfur-penicillin test no tablet) in different formats, have been validated according to international standards. These tests have comparable limits of detection (LODs) to the European Union's maximum residue limits (MRLs) for ten antimicrobial substances. Visual observation and the Delvo Scan system are used to determine the end-points of sample measurements. The assays have demonstrated robustness and resistance to procedural changes (Stead *et al.*, 2018).

While microbial inhibition tests, including the Delvo test, are widely used for screening antibiotic residues in milk due to their reliability and cost-effectiveness, positive samples need to be confirmed and quantified by confirmatory methods such as liquid chromatography-mass spectrometry (LC-MS). Alternative approaches like the yogurt-culture test have also been developed as routine monitoring methods (Gaudin *et al.*, 2018; Jevtusevskaja *et al.*, 2016; Kantiani *et al.*, 2009; Yamaki *et al.*, 2004).

2.12 Technologies of Biosensors in the Determination of Antibiotics in milk

Biosensors are practical tools that utilize transducers, typically employing optical, electrochemical, or piezoelectric mechanisms. Their main purpose is to convert signals originating from biochemical reactions or interactions into measurable outputs. This is achieved by attaching a biomolecule onto the transducer's surface through techniques like physical adsorption, covalent bonding, and cross-linking. Enzymes, antibodies or antigens, and nucleic acid derivatives are the primary biomolecules used for this purpose. These biomolecules play a critical role in imparting specific and selective characteristics to biosensors. Their distinct interactions with complementary components lead to minimal responses to interfering substances (Mehlhorn *et al.*, 2018).

The significance of biosensor devices lies in their capacity to enhance the detection of environmental contaminants, foodborne pathogens, and various clinical conditions. With the additional benefits of cost-effectiveness and efficiency, these devices hold potential for replacing conventional methods in detecting antibiotic residues. In essence, biosensors serve as practical tools that employ transducers to convert biochemical signals into measurable results. Transducers, often working through optical, electrochemical, or piezoelectric means, are responsible for this conversion process. Biomolecules like enzymes, antibodies, and nucleic acids are immobilized on the transducer surface using methods such as physical adsorption or covalent bonding to facilitate this signal transformation. These biomolecules contribute significantly to crucial biosensor traits such as specificity and selectivity. Their unique interactions ensure that undesired substances do not cause significant interference, thereby upholding the accuracy of biosensor readings (Mehlhorn *et al*., 2018).

Biosensors find diverse and impactful applications, encompassing the detection of environmental contaminants, identification of foodborne pathogens, and clinical diagnosis. The advantages of this technology include not just its analytical capabilities but also its cost-effectiveness and efficient operation. Consequently, biosensors hold promising potential to outperform traditional methods, emerging as the favoured choice, particularly in the detection of trace amounts of antibiotics and other substances (Ding *et al*., 2022; Mehlhorn *et al*., 2018).

Food industries, particularly within the dairy sector, testing for residues in raw materials often involves rapid qualitative methods. Nonetheless, there's a simultaneous need for swift and quantitative analysis to ensure adherence to regulated Maximum Residue Limits (MRLs). This demand can be effectively addressed by employing biosensors. These compact devices not only offer quick on-site analysis but also have the potential to integrate with mobile phones, making their application practical and versatile. This adaptability is exemplified by ongoing research into the detection of SARS-CoV-2 (Ding *et al*., 2022; Mehlhorn *et al*., 2018). Consequently, the adoption of biosensor technology presents a viable solution. However, it requires further investment and research to yield a broader array of commercially available devices. Nevertheless, the field holds significant market potential, particularly in the realm of electrochemical devices. These devices are primarily developed for identifying contaminants and assessing toxicity in products. Additionally, they prove invaluable for quantifying carbohydrates resulting from fermentation processes, detecting food adulteration, and evaluating biochemical oxygen demand. Notably, most studies

leverage either optical or electrochemical transducers as their fundamental technology (Mehlhorn *et al*., 2018).

2.12.1 Optical biosensors

Optical transducers stand out as primary choices in biosensor development due to their attributes such as low detection limits, simplified procedures, cost-effectiveness, wide detection range, versatile transducer configurations, compositional adaptability, and potential for enhancing sensitivity and selectivity. Many optical systems feature aptamer-based biosensors that function as assays conducted in solution rather than on transducer surfaces. This approach garners attention due to the ease of modifying aptamers in aqueous solutions compared to immobilizing them on solid transducers. These systems represent an advancement in biosensor technology, alleviating challenges in biomolecule modification while retaining activity. This, in turn, maintains or even enhances the analytical advantages applicable to new devices (Ding *et al*., 2022; Mehlhorn *et al*., 2018).

Surface plasmon resonance (SPR) serves as a prominent approach utilized in optical biosensors for detecting milk residues. A case in point is Altintas' utilization of SPR to identify vancomycin. This was achieved using a gold chip modified with a nano-molecularly imprinted polymer (nano MIP), resulting in a limit of detection (LOD) of 17.7 ng/mL. Another frequently employed technique is fluorescence spectroscopy (FS). Nevertheless, unlike SPR-based biosensors, spectroscopic systems are typically employed in solution-based assays where the biological interaction components are situated within the bulk solution, as opposed to being on a transducer surface (Mehlhorn *et al*., 2018).

Ding *et al*. (2022) applied fluorescence spectroscopy (FS) to identify kanamycin residues. They utilized an aptasensor, achieving a limit of detection (LOD) of 8.72 ng/mL. Their approach involved utilizing carbon dots through a competitive quenching technique, wherein an aptamer interacts with a gold nanoparticle. This interaction changes the absorption spectrum of the nanoparticle aggregates, preventing carbon dot quenching. This concept of gold nanoparticle aggregation can be employed without additional markers, relying on color changes due to aggregation. Zhang *et al.* (2020) demonstrated such approaches for tobramycin and gentamicin determination, respectively. The latter used a paper-based biosensor with a cell phone camera and image software to analyze RGB color, achieving an LOD of 143.28 ng/mL.

The challenge of multiple antibiotic residues in milk due to cattle treatment and synergistic effects is addressed by optical biosensors. For instance, Tuei *et al.* (2021)

employed near-infrared fluorescence for simultaneous detection of cefquinome, tetracycline, enrofloxacin, and sulfadimine. Wu *et al.* (2019) developed a transmission spectrum prototype to detect kanamycin, ampicillin, oxytetracycline, and sulfadimethoxine, potentially applicable as an on-farm device. Wang *et al.* (2013) achieved the lowest LOD for multiple residues using an optical fiber-mediated immunosensor multiplexed assay for chloramphenicol, sulfadiazine, and neomycin, with a LOD of 2.86×10^{-3} ng/mL.

The commonly used techniques, specifically SPR and FS, demonstrated both low detection limits and the ability to identify multiple residues within a single device. However, the lowest limit of detection (LOD), spanning optical, electrochemical, and other systems, was achieved by Van Dijk (2015) through the utilization of conjugated commercial microplates in an aptasensor for kanamycin detection. This system exhibited the capability to identify concentrations below 5.0 × 10-8 ng/mL. Significantly, these approaches exhibited efficacy in detecting commercial milk samples with remarkable recovery rates, underscoring the potential for biosensors to surpass standard assays like ELISA (Van Dijk, 2015).

Gold electrodes or nanoparticles are commonly favored in optical systems due to the strong sulfur-gold interaction or quantum optical properties of nanoparticles upon aggregation. Nevertheless, a variety of materials were also employed, including silicon, silver, paper, magnetic beads, and carbon derivatives. Tariq *et al.* (2020) utilized molybdenum sulfide nanosheets as a substrate for kanamycin detection. Surre *et al.* (2018) developed a lateral flow immunoassay test strip employing fluorescence microspheres to determine chloramphenicol, florfenicol, and thiamphenicol. This study's successful application to blind milk samples with high accuracy was notable. Tariq *et al.* (2020) used lateral flow immunoassays with test strips and raw milk samples.

In their work, Wang *et al*. (2013) employed magnetic nanoparticles to immobilize a cefquinome aptamer through a streptavidin-biotin interaction. This aptamer was hybridized with a carboxyfluorescein-labeled probe. The residue within the sample exhibited a strong interaction with the aptamer, leading to the release of the labeled probe into the solution. Following this, magnetic separation was conducted using a neodymium magnet, resulting in the collection of the non-magnetic supernatant. This supernatant was subsequently analyzed using fluorescence spectroscopy (FS). The utilization of magnetic separation has gained increasing prominence in innovative immunoassays and has been applied across various studies, including those involving SARS-CoV-2 (Ding *et al*., 2022). All of these studies incorporated some form of milk, whether commercial, obtained directly from companies, skimmed, or raw, as detailed in their respective papers. The residues most frequently

evaluated included (from the least) tetracycline (3), enrofloxacin (3), florfenicol (3), chloramphenicol (4) and kanamycin (9). (Ding *et al.,* 2022).

2.12.2 Electrochemical biosensors

Electrochemical biosensors, alongside their optical counterparts, are notable both in terms of quantity and sensitivity. Within this domain, voltammetric techniques and electrochemical impedance spectroscopy (EIS) emerge as pivotal choices for device development. Out of the ten instances of lower limits of detection (LODs) that were identified, six instances utilized differential pulse voltammetry (DPV), three instances employed electrochemical impedance spectroscopy (EIS), and one instance utilized a photoelectrochemical system. A consistent pattern is evident in the choice of electrodes, where gold electrodes (8) and carbon derivatives such as screen-printed electrodes (11) and glassy carbon electrodes (8) were the preferred materials (Mehlhorn *et al*., 2018).

Electrochemical techniques yielded more research papers than optical methods. The accessible and cost-effective nature of electrochemical equipment likely contributes to researchers' preferences. This trend is reinforced by the possibilities offered by label-free techniques like EIS, enabling analyte determination through changes in charge transfer resistance on the electrode surface. An illustrative example is the work by developed a label-free biosensor utilizing an aptamer-modified glassy carbon electrode (GCE) to impedance assay kanamycin. The electrochemical response is generated solely from the electronic transfer hindrance of Fe $(CN)_6^{3-/4-}$, as the aptamer undergoes folding upon exposure to the antibiotic. This response mechanism does not require the presence of an optical or electrochemical marker.

Electrochemical impedance spectroscopy (EIS) has emerged as a prominent technique for achieving low limits of detection (LOD) in electrochemical biosensors. This is evident in the determination of penicillin-G (2.7×10^7 ng/mL) and penicillin (0.849×10^3 ng/mL). In an innovative approach, Rosati *et al*. (2019) utilized EIS on various low-cost substrates, including glossy paper, commercial glossy paper, office paper, and PET polymer modified with silver nanoparticles ink, obtained from a conventional office printer. This inkjet-printed, label-free electrochemical aptasensor was developed for ampicillin detection. Although the LOD for antibiotic detection in milk was relatively high, the system shows potential for enhancement in subsequent studies. Furthermore, it offers a compact, straightforward, and rapid biosensing approach, which is rarely observed from a commercial standpoint during initial research investigations (Mehlhorn *et al.,* 2018).

Accompanying EIS, amperometry emerges as a recurrently employed technique for biosensor development. It is noteworthy to acknowledge the persistent efforts of specific research groups dedicated to advancing science, human resource training, benefiting the food industry, and serving consumers. The team led by Mehlhorn has devoted years to crafting electrochemical biosensors for antibiotic detection in milk samples. The research group's systems have undergone advancements in electrode modifications while maintaining a consistent amperometric approach. These advancements involve indirect detection through competitive assays between the target analyte and an HRP-labeled analyte (or analog). The presence or absence of HRP is ascertained through electrochemical reactions within a solution containing hydrogen peroxide and hydroquinone. The significance of these efforts is highlighted by the collaboration of researchers with Mehlhorn. They built upon the previous system and introduced innovation by integrating a controlled biofuel cell for the determination of sulfapyridine. The remarkable analysis of milk samples and the noteworthy sensitivity achieved illustrate advancements in the development of a point-of-care device for rapid and reliable measurements (Mehlhorn *et al*., 2018).

Differential pulse voltammetry (DPV) emerges as a crucial technique in biosensor development, demonstrating sensitivity comparable to square wave voltammetry. In a multifaceted approach, Zhang *et al*. (2013) designed an aptasensor for kanamycin determination, employing intricate biochemical reactions both on the electrode and in solution. The researchers modified a gold electrode with a thiol-modified hairpin probe in a stem-loop structure (HP2). In solution, the antibiotic interacted with another hairpin probe (HP1), exposing the hairpin stem for enzymatic action by Klenow fragment polymerase, primer, and nicking endonuclease. This sequence generates single-stranded DNA, altering the HP2 structure and creating residual single-stranded DNA on the electrode surface. Upon introducing hairpin 1 (H1) and hairpin 2 (H2), a hybridization chain reaction is triggered. The indirect electrochemical detection utilizes the signal of the DNA intercalator, methylene blue. In the absence of the antibiotic, the cascade of biochemical reactions does not occur, resulting in a weak or nonexistent methylene blue signal.

Aptamers are the predominant choice in biosensor development, synthesized through the systematic evolution of ligands by exponential enrichment (SELEX) method. These molecules exhibit an affinity to the analyte comparable to the antibody-antigen interaction, enhancing selectivity. Aptamers used in antibiotic determination often originate from SELEX or SELEX-derived experiments. For example, a comprehensive study encompassing aptamer selection through SELEX to application in an electrochemical biosensor for penicillin G

detection was conducted. After selection and screening, screen-printed carbon electrodes (SPCE) were modified with the aptamer displaying the best retention rate in chromatographic assays. With remarkable recovery rates in milk samples, the designed aptamer provides a novel avenue for subsequent biosensor development (Ding *et al*., 2022).

Perry and Grace (2009) introduced a captivating method for modifying electrodes, offering an alternative to the gold-sulfur interaction. This novel technique utilized diazonium salts. A similar methodology was also pursued by Sharma *et al*. (2018). This approach involved the introduction of functional groups onto the electrode's surface. This, in turn, facilitated biological interactions through activation using EDC/NHS or glutaraldehyde. The significance of these strategies lies in their ability to maximize the immobilization of biological materials while preventing any potential leaching. Furthermore, gold or silver nanoparticles as well as carbon nanotubes find extensive utility. Their roles encompass various functions such as bridging the connection between the electrode and biomolecule, augmenting the electrode's surface area, and amplifying the electrochemical signal.

Li *et al*. (2014) show cased an inventive approach in the advancement of biosensors. They conducted modifications on a screen-printed carbon electrode (SPCE) with a combination of carbon nanofibers, silver nanoparticles, carbon nanotubes, and two distinct complementary strands. These strands were designed to match the kanamycin aptamer and the streptomycin aptamer, both linked to a polymer. This intricate setup enables the concurrent detection of these substances by modifying each unique aptamer with a specific quantum dot. To illustrate, the kanamycin aptamer was linked to CdS quantum dots, while the streptomycin aptamer was connected to PbS. These aptamers selectively hybridize with the corresponding complementary strands already affixed to the electrode's surface.

When both kanamycin and streptomycin antibiotics are present in the solution, they displace the quantum dot-aptamer complexes from the electrode surface. This leads to an increase in the electrochemical signal of Cd^{2+} and Pb^{2+}. Because these cations display distinct voltammetric characteristics, it becomes possible to identify the presence and quantity of both antibiotics in a single measurement. This research ushers in new possibilities, as various metal-based quantum dots can be employed alongside different aptamers. This innovative approach paves the way for creating devices capable of multiple drug determinations using individual or arrays of electrodes (Li *et al.,* 2014).

Electrochemical systems have also enabled simultaneous determinations or the use of biosensors for multiple analyte assessments. For instance, Li *et al*. (2014) developed systems to determine at least two antibiotics. Additionally, Perterson & Kaur (2018) devised

biosensors for three or more residues. Among the main evaluated residues, kanamycin (8) took the lead, followed by chloramphenicol (5), penicillin-G (5) and ampicillin (6). However, electrochemical systems exhibited greater versatility than optical systems, with a wide array of different analytes (24) being explored.

2.12.3. Other biosensors

Beyond optical and electrochemical biosensors, several other significant systems, although fewer in number, have been utilized in the development of biosensors for antibiotic residue detection. Ding *et al.* (2022) developed a biosensor utilizing an acoustic wave technique based on frequency change. They employed a gold electrode modified with penicillin-G antigen. The researchers achieved a limit of detection (LOD) of 2.20 ng/mL in commercial milk samples. Raffay *et al.* (2022) also created a nanoscale LOD piezoelectric system. They employed a quartz crystal coated with a gold film to immobilize streptomycin antibodies. An interesting and recurring system was employed by Mehlhorn *et al.* (2018), involving smartphone camera images to analyze ciprofloxacin residues on a commercial chip. The presence of the analyte induces alterations in light intensity due to interaction with bacteria. Although categorized as an optical system, this biosensor stands out due to being the only bacterial biosensor among the array of immunosensors and aptasensors evaluated.

The biosensors discussed in this paper encapsulate a decade's worth of proof-of-concept studies that address the escalating concern over antibiotic usage in dairy cattle. This concern has created a pressing need for swift, on-site qualitative and quantitative analyses. Despite the excellent quality of these works, the adoption of these devices within the dairy sector remains limited, possibly due to the well-established reliability of immunoassays. Across the board, numerous antibiotic residues were evaluated, with an emphasis on kanamycin, chloramphenicol, ampicillin, and penicillin-G (Ding *et al.*, 2022).

Kanamycin, an aminoglycoside drug discovered in 1957, was previously used to treat tuberculosis in humans but was discontinued due to its toxic effects and limited action spectrum. Presently, it is authorized for treating mastitis in cows, although its use is not allowed in food-producing animals in the USA. While no commercial biosensor for kanamycin was identified online, the findings from Table 2, Table 3, and Table 4 reveal several promising studies featuring low limits of detection, miniaturization prospects, wide linear ranges, and cost-effectiveness. These studies present a foundation for further development into reliable and efficient market-ready products (Mehlhorn *et al.*, 2018).

Certain biosensor designs still rely on costly reagents and multistep processes to enhance sensitivity and selectivity, potentially introducing non-appealing expenses for

farmers. Nevertheless, innovative researchers are exploring alternatives, including simpler modifications and analyses, such as employing image processing software for cell phone images or utilizing common materials like office paper for electrodes. Other examples include the use of inexpensive pencil carbon graphite leads, screen-printed electrodes, and genetic engineering for aptamer selection and production (SELEX). A range of options exists to advance existing systems toward commercialization (Ding *et al*., 2022; Mehlhorn *et al*., 2018).

Chloramphenicol, discovered in 1947 and widely used to treat conjunctivitis, has also demonstrated human toxicity, including aplastic anemia and the "gray baby" syndrome (Ding *et al*., 2022). While proof-of-concept studies for chloramphenicol exhibit various electrode configurations, modifications, and detection techniques, the availability of well-established commercial immunoassays for the dairy industry has influenced investment priorities, potentially hampering the uptake of novel devices or technologies (Ding *et al*., 2022; Mehlhorn *et al*., 2018).

The journey of studying and developing biosensors spans decades, resulting in a wealth of knowledge. To move beyond proof-of-concept, researchers must now focus on the practical application of these systems, transforming them into point-of-care devices that facilitate on-site measurements with minimal instruction and easy interpretation, all while maintaining cost-effectiveness (Ding *et al*., 2022; Mehlhorn *et al*., 2018).

The concern regarding antibiotic residues, particularly those that have not been addressed by biosensor studies, highlights a disconnection between the knowledge held by those overseeing the raw products, often ranchers, and the absence of incentive-based policies aimed at enhancing the overall quality of the end products within the dairy industry. This issue becomes particularly evident with the antibiotics most frequently utilized for cattle, which coincidentally tend to be those least covered by biosensor research. This disconnect impacts small-scale producers, leading to the formulation of mixed treatments that frequently lack synergistic effects and are unsuitable for animals producing milk (Ding *et al*., 2022; Mehlhorn *et al*., 2018).

Furthermore, the potential for human errors in accurately determining the appropriate timing for discarding milk from treated animals' results in the inadvertent distribution of contaminated milk within the industry. Ensuring accurate milk assessment is of utmost importance, as the industry frequently imposes fines on producers based on the amount of contaminated milk present in transport truckloads. This not only leads to financial losses but also jeopardizes the livelihoods of workers. Such circumstances could be alleviated through

the implementation of pre-loading measurements, enabling proactive identification and mitigation of contamination issues before loading occurs

(Ding *et al*., 2022; Mehlhorn *et al*., 2018).

Hence, the dairy sector necessitates new technologies and methodologies to optimize efficiency in production, elevate quality of the product, and reduce losses in adherence to existing legislation. The studies examined here showcase the turnaround of these systems over time, the integration of new pragmatic considerations like aptamers and immunoassays, and the high likelihood of discovering competitive and dependable commercial devices in the near future.

2.13 Summary and Study Rational (The Study Gap)

In summary, the presence of antibiotic residues in milk poses significant risks to consumer health and dairy industry production. Antibiotics used in veterinary medicine can result in residues in milk, affecting its quality and safety (Asredie & Engdaw, 2015; Sachi *et al.*, 2019). The detection of antibiotic residues is crucial due to their potential health hazards and the emergence of antibiotic resistance (Iwu *et al.*, 2020; Imperial & Ibana, 2016). However, current detection methods, such as high-performance liquid chromatography (HPLC), are expensive and not easily accessible to small-scale farmers (Bitas & Samanidou, 2018). Thus, there is a need for a cost-effective and user-friendly method for detecting antibiotic residues in milk.

The widespread use of antibiotics in livestock leads to antibiotic residues in milk, which can have adverse effects on consumer health and the production of dairy products (Alsager *et al.*, 2018; Haas *et al.*, 2019). Antibiotic residues may cause allergies, intestinal alterations, and the development of multidrug-resistant bacteria (Alsager *et al.*, 2018). Furthermore, the presence of antibiotic residues in milk can disrupt the normal microflora and impact the manufacturing processes of dairy products like yogurt and cheese (Haas *et al.*, 2019). The excessive use of antibiotics in treating intramammary infections in dairy herds can compromise milk quality and consumer health (Sharun *et al.*, 2021). Therefore, effective detection methods are crucial for monitoring and controlling antibiotic residues in milk.

The global usage of antibiotics in livestock contributes to the release of unaltered antibiotics into the environment, leading to the emergence of antibiotic resistance (Iwu *et al.*, 2020; Rawson *et al.*, 2017). Antibiotic resistance poses a significant public health problem worldwide (Wurpts *et al.*, 2019). Although detection methods for antibiotic residues exist, they are often expensive and not suitable for small-scale farmers (Ahmed *et al.*, 2020;

Majdinasab *et al.*, 2020). Hence, there is a need for an affordable and user-friendly method that can be easily implemented by farmers or milk graders in the field (Ahmed *et al.*, 2020). Developing a quick, low-cost, and efficient method for detecting antibiotic residues is essential to ensure consumer safety and enable easy monitoring of milk quality.

In conclusion, the presence of antibiotic residues in milk poses risks to consumer health and dairy industry production. The current detection methods, such as HPLC, are expensive and not easily accessible to small scale farmers. There is a need for a cost-effective and user-friendly method for detecting antibiotic residues in milk. Effective monitoring and control of antibiotic residues are crucial to mitigate the health hazards associated with their presence and to combat the emergence of antibiotic resistance. Developing such a method would enable timely detection and ensure the safety and quality of milk consumed by the public.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Site

The study took place in Nakuru County, Kenya, as shown in Figure 3.1, which illustrates the study area. The physical experiment was conducted at Olenguruone Farmers' Cooperative in Nakuru County. The laboratory work, on the other hand, was carried out at both the laboratory of Olenguruone Farmers' Cooperative and the laboratory of Happy Cow Ltd.

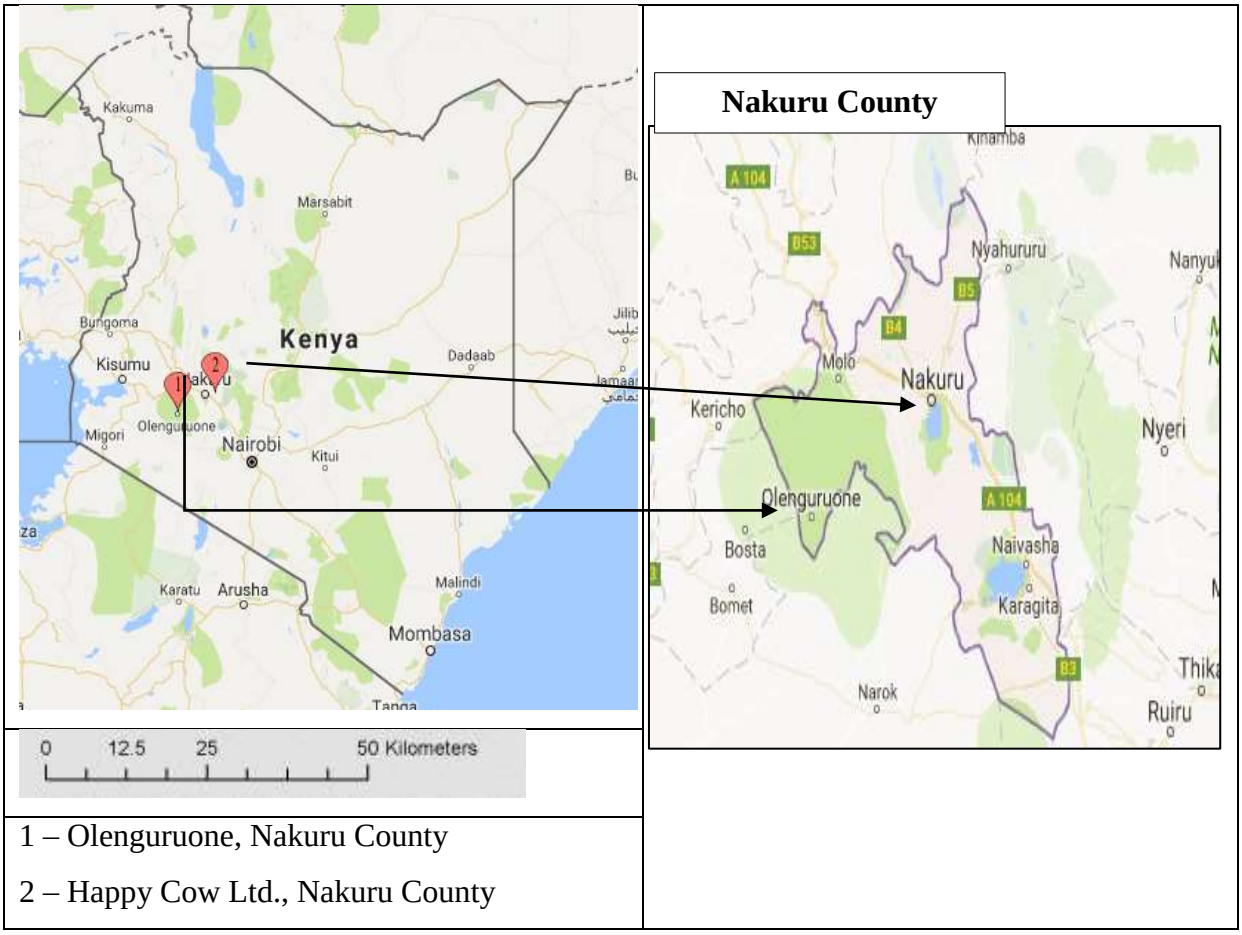


Figure 1: Map of study site

The study took place from May 2020 to November 2021, with two parts conducted at Olenguruone Dairy Farmers Cooperative Society in Nakuru County. The selection of Olenguruone Dairy Cooperative was based on its well-equipped laboratory and its suitability for the study area. Additionally, the cooperative allowed access to their farmers' information and animals. Most of the farmers associated with Olenguruone dairy were small-scale producers, with an average daily cow milk production of 5 to 6 liters. During the study

period, the cooperative had 1,800 active members and collected approximately 8,000 liters of milk per day. Various modes of milk collection transport were used, including motorbikes, vehicles, donkey carts, and individual farmers' deliveries. The study specifically took place at the main cooling plant, which housed the milk quality control laboratory.

Olenguruone Settlement is located in Rift Valley Province, Kenya, within the Africa/Middle East time zone. Its geographical coordinates are approximately 0°37'0" S and 35°37'60" E, measured in degrees, minutes, and seconds. Its milk catchment and collection area is in Nakuru County and part of Narok and Bomet Counties of Kenya.

The third part of the study was conducted at the milk quality control laboratory of Happy Cow Ltd., a privately-owned family business specializing in the production of high-quality value-added dairy products, primarily cheese and yoghurt. The company primarily sources its raw milk from dairy smallholder cooperatives. The laboratory at Happy Cow Ltd. is accredited by the Kenya National Accreditation Services (KENAS) and authorized to conduct various testing parameters, including antibiotic residue testing.

Happy Cow Ltd. is located in Nakuru County, Kenya, with geographical coordinates approximately 0°17'25.4" S and 36°06'44.8" E, measured in degrees, minutes, and seconds.

3.2 Selection of experimental animals and treatment

A medium-scale farm situated in Olenguruone was selected and utilized for the purpose of this study. The farm housed a total of 14 lactating animals, comprising both Friesians and Ayrshire breeds, which was a crucial requirement for the study. The daily milk production per cow on the farm ranged between 5 and 9 kg/day. The selection criteria for the participating animals involved considering their breed type (pure Friesians or Ayrshires), lactation stage, the presence of four functional udder quarters, and the absence of visible signs of mastitis.

Composite milk samples were collected from all teats of each cow and subsequently taken to the laboratory for analysis. The laboratory findings revealed that all the cows had subclinical mastitis, with the identified causative microorganisms being *Staphylococcus aureus* and *Enterobacteriaceae species*.

To address the infection, six randomly selected cows (3 Friesians and 3 Ayrshires) underwent treatment under the supervision of a qualified veterinary officer. The treatment involved intramammary infusions performed on each cow's four teats. The administered drug consisted of Procaine Penicillin G (60mg), Streptomycin Sulphate (100mg), Neomycin Sulphate (100mg), and Prednisolone (10mg). This treatment was administered only once on

the first day. Simultaneously, on the same day and time, the cows received injections of a drug comprising 120 mg Procaine benzylpenicillin and 200mg Dihydrostreptomycin, totaling 30mls. These injections were administered for three consecutive days.

3.3 Milk sampling

The sampling procedure employed in this study followed the guidelines outlined in ISO 707 and IDF 50, 2008. Prior to initiating the treatment, a pooled antimicrobial-free (negative) milk sample of 5 liters was collected separately from three Friesian cows and also from Ayrshire cows. This approach was adopted to mitigate individual variations in milk composition. Throughout the three days of treatment, three days of the withdrawal period, and two days following the completion of the withdrawal period, similar sampling methods were employed for both breeds. In total, 18 samples were collected, with six samples consisting of negative (antibiotic-free) milk taken on the first day before treatment and the two days after the completion of the withdrawal period. All the other samples yielded positive results. To ensure sample integrity, these samples were divided into smaller quantities (100mls) and stored under frozen conditions below -20°C.

3.3.1 Preparation of milk samples

Before analysis, the samples (both positive and negative) were brought to room temperature and were properly mixed to obtain an even and homogenously distributed sample. Then the homogenous sample was distributed to different clean test tubes using a pipette for analysis as was required.

3.4 Experimental design

In this study, pooled milk samples known to be either beta-lactam antibiotic positive or negative were utilized in all experiments. Three laboratory experiments were conducted, which included: modifying the ingredients of the HDBT (Hardy Diagnostic Beta-Lactam Test) reagent to enhance its performance, determining the optimal mixing ratios (milk: reagent) for improved results, and investigating the impact of breed on the test method outcomes. Sensory evaluation involving trained panelists was employed for each experiment.

The modification of reagent ingredients for test method development utilized a Randomized Complete Block Design (RCBD) with three replications. The establishment of mixing ratios and the examination of breed effects on the test method results employed a Completely Randomized Design (CRD) with three replications.

To compare various kits, a total of twenty-eight distinct samples with an approximate volume of 50ml each were prepared for analysis. Out of these samples, there were 18 that tested positive for beta-lactam antibiotics. These positive samples encompassed 8 milk samples devoid of antibiotics, deliberately spiked with known concentrations (ranging from 1%, 5%, 10%, 15%, 20%, 25%, 50%, to 75%) of beta-lactam residues, and 10 milk samples known to be free of antibiotics, serving as the negative control group. Before commencing the experiment, all samples were assigned numerical codes to ensure randomization. Subsequently, the samples were promptly analyzed following their preparation using the five different test methods mentioned earlier. The analysis procedures as outlined in the technical bulletins and manufacturer's instructions of the respective test methods were meticulously adhered to. Detailed information regarding the reduction of milk residue concentrations over time subsequent to beta-lactam antibiotic treatment, considering both intramuscular injections and intramammary infusions can be found in Table 1 of the study.

Table 1: Milk residues concentrations (µg/L) in hours following intramammary infusions intramuscular injections in lactating and animals

Time (hrs.)	Intramuscular injections	Intramammary infusions
24	27	289,000
48	4	9260
72	1	551
96	0	44

Source: EFSA *et al.* (2017)

3.5 Modification of HDBT Ingredients in Test Method Development

The HDBT utilized five (5) specific ingredients, namely phenol red, trisodium phosphate, trisodium citric acid, sodium chloride and penicillin and as outlined in the test description. The components were procured from Chemoquip Limited in Nairobi. Prior to their utilization, the certificates of analysis and seals of these components were meticulously examined to confirm their authenticity. The formulation of the reagent, as outlined in the HDBT test, is provided in Table 2. The components were thoroughly blended with 1 liter of distilled water. The first experiment focused on utilizing the HDBT reagent (reagent 1) in its original composition as detailed in Table 2. In this experiment, equal quantities of milk samples (both beta-lactam positive and negative) and the reagent were combined, and any colour differences were observed.

Table 2: The composition of Hardy Diagnostic Beta-Lactamase Test reagent

S/no.	Ingredient	Amount (g)/L of H_2O
1.	Penicillin	15.0
2.	Sodium Chloride	5.0
3.	Trisodium Citrate	1.5
4.	Trisodium Phosphate	0.3
5.	Phenol Red	0.018

In the second experiment, four reagents were prepared using the ingredient amounts specified in Table 2, with one ingredient being excluded at a time, as indicated in Table 3. This resulted in the creation of four distinct reagents, namely reagent 2, reagent 3, reagent 4, and reagent 5.

Reagent 2 consisted of trisodium phosphate, sodium chloride, penicillin, and phenol red, while excluding trisodium citrate. Reagent 3 included trisodium citrate, sodium chloride, penicillin, and phenol red, but excluded trisodium phosphate. Reagent 4 comprised trisodium citrate, trisodium phosphate, penicillin, and phenol red, excluding sodium chloride. Lastly, reagent 5 contained trisodium citrate, trisodium phosphate, sodium chloride, and phenol red, but excluded penicillin.

Each reagent was mixed with equal amounts of known beta-lactam positive and negative milk samples, and the resulting colour differences were observed. Table 3 indicates the composition of each reagent, where a checkmark (√) signifies that the ingredient was included in the specified amounts from Table 2, while a dash (-) indicates that no amount of that particular ingredient was used.

Table 3: Experiment 2 reagent composition

Reagent code	Trisodium phosphate	Sodium chloride	Penicillin	Phenol red	Trisodium citrate
2	√	√	√	√	-
3	-	√	√	√	√
4	√	-	√	√	√
5	√	√	-	√	√

In the third experiment, the reagents were prepared using three times the amount of each ingredient compared to the previous experiments. This led to the creation of five different reagents, namely reagent 6, reagent 7, reagent 8, reagent 9, and reagent 10.

Reagent 6 contained an excess of penicillin, reagent 7 had an excess of trisodium phosphate, reagent 8 had an excess of sodium chloride, reagent 9 had an excess of phenol red indicator, and reagent 10 had an excess of trisodium citrate. Each reagent was mixed with equal amounts of known beta-lactam positive and negative milk samples to observe any colour differences. In the fourth experiment, the specific composition of ingredients that would result in significant changes was investigated at various levels. The quantities prepared in the third experiment are provided in Table 4, indicating the amounts used for each ingredient.

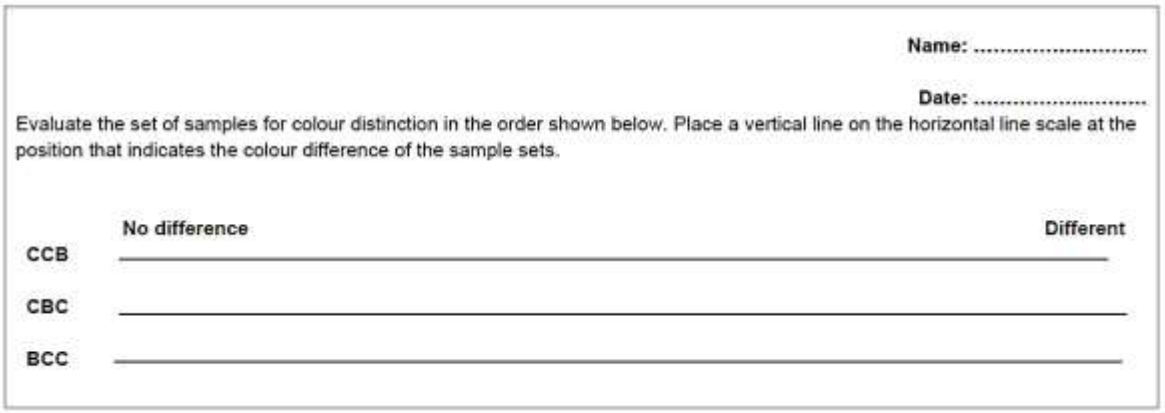
Table 4: Experiment 3 reagent composition

Reagent code	Trisodium phosphate	Sodium chloride	Penicillin	Phenol red	Trisodium citrate
6	0.3	5	45	0.018	1.5
7	0.9	5	15	0.018	1.5
8	0.3	15	15	0.018	1.5
9	0.3	5	15	0.054	1.5
10	0.3	5	15	0.018	4.5

In the four experiments, a total of ten trained panelists were involved. They were asked to evaluate the sets of samples and identify the ones that exhibited the largest differences between beta-lactam positive and negative milk samples. The evaluation process utilized both a ranking method and a line scale method, following the approach described by Sharif *et al.* (2017). For the ranking method, the panelists were instructed to assign the highest score of 30 to their most preferred set, indicating the greatest difference between the samples. They were then asked to arrange the sets in descending order based on their perceived differences.

In the line scale method, the panelists were provided with a ballot paper (as shown in Figure 2). They were requested to place a mark on a 15cm line to indicate their perception of the difference between the samples. The line was labeled "no difference" on the extreme left and "different" on the extreme right. Using a ruler, the distance from the "no difference" mark to the point where the panelist placed their mark was measured. These measurements were used in the subsequent statistical analysis. By calculating the means of the measurements obtained from the line scale method, the reagent with the highest mean value was selected as

the most preferred, indicating the set of samples that demonstrated the greatest difference between beta-lactam positive and negative milk samples.


Name: .....

Date: .....

Evaluate the set of samples for colour distinction in the order shown below. Place a vertical line on the horizontal line scale at the position that indicates the colour difference of the sample sets.

	No difference	Different
CCB		
CBC		
BCC		

Figure 2*:* Sample ballot paper for Line scale method issued to panelists

3.5.1 Determination of mixing ratio (reagent vs milk sample)

To determine the appropriate mixing proportions between the reagent and milk, portions of the reagent and milk were mixed at nine different levels. The ratios of reagent to milk that were mixed independently included: 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. For each ratio, a pair consisting of known beta-lactam positive and negative milk samples was prepared, and observations were made.

The selection of the best ratio, which would effectively differentiate the colours between the positive and negative samples, was carried out by 12 trained panelists using the ranking and line scale methods described earlier. The panelists assessed each ratio and ranked them based on their ability to distinctly differentiate the colours of the samples. Additionally, they used the line scale method to mark their perceived differences on a 15cm line.

The ratio that obtained the highest ranking and had the greatest mean value, based on the line scale method, indicating the largest difference between the samples, was considered the best ratio. This selected ratio was then applied in subsequent experiments as the optimal mixing proportion between the reagent and milk.

3.5.2 Determination of breeds effect on the test method outcome

An investigation was conducted to determine if the test method was influenced by different breed types, specifically Friesians and Ayrshires. Pooled raw milk samples from three cows of each breed were collected over the course of nine analysis days. The reagents, as modified in section 3.5.1, and the established mixing ratio from section 3.5.1 were used in these experiments.

The sensory analysis involved 10 trained panelists who utilized the line scale method, as described earlier, to evaluate the samples. The measurements obtained from the panelists' assessments were subjected to statistical analysis to analyze the data. The means of the measurements were then used to determine any observed differences between the two breed types.

By comparing the means obtained from the sensory analysis, it was possible to assess whether there were significant differences in the test method results between the Friesian and Ayrshire breeds. This investigation aimed to identify any potential variations in the test outcomes based on breed type.

3.6 Determination of the reagent's shelf life

The reagent, prepared according to the established composition in section 3.5, was subjected to storage under four different conditions: frozen (below 0°C), refrigerated (below 6°C), at room temperature, and under direct sunlight. Analysis was conducted using known beta-lactam positive and antibiotic-free raw milk samples, utilizing the freshly prepared reagent on the first day. The mixing ratio determined in section 3.5.1 was applied during this analysis.

On the second day, analysis was carried out using the reagents from the four storage conditions, along with a freshly prepared reagent, in conjunction with positive and negative raw milk samples. The freshly prepared reagent served as the control in the experiment. The line scale method was employed to quantify and compare the results obtained, based on the observations of colour changes made by a panel of 10 trained individuals.

By assessing the colour changes observed using the line scale method, the panelists were able to compare the different reagents from the various storage conditions and determine any variations in their performance. This investigation aimed to evaluate the effect of different storage conditions on the reagent's ability to differentiate between positive and negative raw milk samples.

3.7 Determination of Sensitivity and Specificity of Developed Test

The sensitivity and specificity of the developed test method were assessed by conducting a comparative analysis with several rapid antibiotic residue test kits that are commonly found in the Kenyan market. These kits, namely Delvo test Fast BL, Delvo Sulphadiazine Penicillin No Tablet (SPNT), Mtusbio beta-lactam BLQ Rapid Test Kit, Ringbio beta-lactam, tetracycline, sulfa drugs, and BTS 3 in 1 TriTest S., are widely available and widely used for detecting antibiotic residues in different samples.

By comparing the performance of the developed test method with these established kits, the study aimed to evaluate how well the developed method can detect positive samples (sensitivity) and accurately identify negative samples (specificity). This investigation was conducted to determine the efficacy and reliability of the developed test method in comparison to other commercially available options in the Kenyan market. The goal was to provide a comprehensive understanding of the strengths and weaknesses of the developed test method in relation to its counterparts, thereby contributing to the overall assessment of its effectiveness in antibiotic residue detection.

3.7.1 Delvo Sulphadiazine Penicillin No Tablet (SPNT) (Delvo SPNT).

The analysis method employed in this study was AOAC 982.18, which is outlined in the technical bulletin of the Delvo test. This particular test is categorized as a broad-spectrum inhibitor test within the field of microbiology. It is designed to detect not only antibiotic residues but also other inhibitory substances that may be present in milk samples. The test utilizes the microorganism *Bacillus stearothermophilus* due to its high sensitivity to a wide range of antibiotics (Abebew *et al.,* 2014).

The test ampoules used in this analysis contain selected nutrients and a pH indicator called bromocresol purple. To perform the test, 0.1 ml of the milk sample is directly added to the ampoules, followed by an incubation period of 3 hours at a temperature of 64 ± 0.5°C. During incubation, the metabolic activity of the microorganism leads to a change in pH, resulting in a colour transformation of the agar in the ampoules from purple to yellow. This colour change indicates the absence of inhibitory substances in the sample. Conversely, if the sample contains a sufficiently high concentration of inhibitory substances, the colour of the agar will remain purple (Stead *et al.,* 2008). The interpretation of the test results was performed according to the instructions provided by the manufacturer of the Delvo test. These instructions outline the criteria for determining whether a sample is positive or negative for the presence of inhibitory substances based on the observed colour change in the ampoules.

3.7.2 Delvo test Fast BL (Delvo BL).

This testing method utilizes lateral flow technology, which is a one-step receptor assay, to detect beta-lactam residues of antibiotics in raw cow milk. The analysis procedure follows the guidelines outlined in the Delvo test technical bulletin specific to this test. The method is designed to be sensitive to a wide range of drugs containing a beta-lactam ring, including various types of penicillin (both extended and narrow spectrum), beta-lactamase

resistant and sensitive penams, and cephalosporins of different generations (1st, 2nd, 3rd, 4th, and 5th). The test has a high confidence level of at least 95% in detecting positive samples with Penicillin residues in raw milk. The detection limits for specific antibiotics have been determined as 4µg/L for amoxicillin, 3µg/L for ampicillin, and 3µg/L for penicillin. The test kit consists of ampoules, disposable pipettes, and test strips, all of which are used in combination for the analysis. The entire process, including analysis and reading of results, takes approximately 7 minutes.

To conduct the analysis, 0.15ml of raw milk is added to the ampoule, and the contents are thoroughly mixed until fully dissolved. The ampoule is then incubated at a temperature of 64°C ±2°C for 2 minutes, followed by the insertion of the test strip, which is further incubated for an additional 3 minutes. After the total incubation period of 7 minutes, the results are read and interpreted according to the instructions provided in table 5.

3.7.3 Mtusbio Beta-lactam BLQ) Rapid Test Kit (Mtusbio).

The test kit utilized in this study employs a competitive colloidal gold immunoassay method to detect the presence of beta-lactam antibiotics in raw cow milk, specifically targeting the beta-lactam family. The kit was designed to determine whether the concentration of beta-lactam antibiotics in the milk is above or below the tolerance levels. The detection limit of the test kit is 4µg/L for penicillin, ampicillin, and amoxicillin.

During the analysis process, a volume of 200µl of the milk sample was added to the ampoule containing the reagent. The contents of the ampoule are thoroughly mixed for 5 minutes until the milk sample turns pink, indicating the completion of the dissolution process. Subsequently, the test strip was inserted into the ampoule, and after an incubation period of 5 minutes, the results are read.

The interpretation of the test results was carried out according to the instructions provided in table 5, which outlines the different visual indicators or colour changes that correspond to specific concentration levels of beta-lactam antibiotics in the milk sample.

3.7.4 Ringbio Beta-lactam, tetracycline, sulfa drugs, BTS 3 in 1 Tri Test S (Ringbio).

The test under investigation was designed to detect the presence of beta-lactam, tetracycline, and sulfonamide antibiotic residues in milk. It is a receptor assay that relies on the utilization of highly specific antibodies and capture proteins that have a strong affinity for the targeted antibiotics. This allows for the accurate identification of residues without the need for any specialized instruments. The test is conveniently prepared and does not require

an incubation period, providing results within a short timeframe of 6 minutes. The limit of detection for penicillin, amoxicillin, and ampicillin is 4µg/L.

During the analysis process, a volume of 200µl of the milk sample was added to a micro well. The sample and reagent in the well are mixed thoroughly by repeatedly absorbing and releasing the solution five times. This ensures complete and uniform mixing, resulting in a pink-coloured mixture. The mixture was then incubated at room temperature for 5 minutes. Subsequently, a test strip was inserted into the well and another 5-minute incubation at room temperature is carried out. After the incubation, the test strip was removed from the well, and the results can be read.

The interpretation of the results was conducted according to the instructions provided in table 5, which outlines the visual indicators or colour changes corresponding to different concentration levels of beta-lactam, tetracycline, and sulfonamide antibiotic residues in the milk sample.

Table 5: Results interpretation for rapid test kits (Delvo BL, Ringbio, Mtusbio)

Colour intensity comparisons	**Result Interpretation**	**Result Analysis**
CL alone	Blank/Valid	The test strips are not faulty/blank sample
TL alone	Invalid	The test is invalid; control line is absent
TL more intense than CL	Negative	The sample contains no antibiotics or contains antibiotics at lower level than the detection limits
TL is similar to CL	Weak Positive	The sample contains antibiotics close to the detection limits
TL is absent or less intense than CL	Positive	The milk sample contains antibiotics above the detection limits

TL- Test Line and CL=Control Line

3.7.5 Developed test method (Ndungu Antibiotic Residues (NAR) test).

The method employed in this test is an acidometric approach that relies on the hydrolysis of the beta-lactam ring by the enzyme beta-lactamase. This enzymatic reaction leads to a decrease in pH due to the production of penicilloic acid. The pH change was detected by the presence of phenol red, which acts as an acid-base pH indicator and undergoes a colour change in response to the altered pH levels.

This test method offers several advantages, including its simplicity and rapidity. It can be conveniently performed at room temperature without the need for complex equipment, electric power source or specialized conditions. The reagent ingredients, as modified according to the procedure outlined in section 3.5, were utilized in the test. During the analysis, observations were made regarding any colour differences exhibited in the reaction mixture, and the corresponding results were recorded for further analysis and interpretation.

3.8 Statistical analysis

The analysis of data concerning the reagent modification and the determination of its shelf life was carried out using the PROC GLM (general linear model) procedure within the statistical analysis system (SAS) version 9.4M6 by SAS Institute Inc. This involved conducting an analysis of variance (ANOVA), with mean separation performed through the least significant difference (LSD) method whenever significant differences were identified.

For evaluating the new method's sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) in comparison to other test methods, true positive and true negative results were organized in tabular form. These data were subjected to analysis using the "diagt" command in STATA version 12. The PPV denotes the proportion of milk samples that tested positive and were indeed positive, while the NPV signifies the proportion of milk samples that tested negative and were indeed negative. Additionally, the agreement between the tested methods was determined using Cohen's kappa coefficient (k), and the association among the methods was assessed through the odds ratio.

3.9 Research Permit and Ethical approval

The study adhered to ethical guidelines, and the necessary ethical clearance and approval were obtained from the Egerton University Ethics Review Committee (EUREC) under approval number EUREC/APP/097/2020, as well as from the National Commission for Science, Technology and Innovation (NACOSTI). Approval was also obtained from the Board of Postgraduate Studies of Egerton University.

Prior to their participation in the study, the purpose and procedures of the research were thoroughly explained to the study participants. Written informed consent was obtained from each participant, ensuring their voluntary involvement in the study. The confidentiality of research information and data was strictly maintained. This was achieved through the use of signed consent forms, secure password-protected computers, and adherence to professional standards of conduct to ensure the privacy and anonymity of the participants.

CHAPTER FOUR
RESULTS

Introduction

The data analysis was conducted using SAS version 9.2.2 to assess the performance of the NAR (Negative and Reversion) test method for detecting beta-lactam antibiotic residues in milk samples. This analysis aimed to evaluate the accuracy and consistency of the NAR test results compared to other detection methods. The data collected from the test samples were processed and analyzed using various statistical techniques available in the SAS software. Specifically, the NAR test results were compared to those obtained from other test methods, such as Delvo (BL and SPNT)/Mtusbio/Ringbio. Various parameters were calculated to evaluate the performance of the NAR test, including sensitivity, specificity, positive predictive value, and negative predictive value. These parameters provided insights into how well the NAR test was able to correctly identify both positive and negative samples for beta-lactam residues.

The odds ratio was computed to assess the likelihood of the NAR test providing accurate results compared to the other methods. Additionally, the Kappa coefficient was utilized to measure the level of agreement between the NAR test and the other methods. This coefficient helped determine whether the level of agreement was beyond what would be expected due to random chance. The SAS software's analytical tools allowed for thorough statistical comparison of the NAR test results with other detection methods. By analyzing these parameters, the study aimed to draw conclusions about the NAR test's reliability, accuracy, and consistency in detecting beta-lactam antibiotic residues in milk samples.

4.1 Developing the Beta-Lactam Detection Test Method

4.1.1 Determination of Amounts of Ingredients for the Developed Test Method

In the initial experiment, when the HDBT reagent was used in its original composition, the colour differences observed were not distinct enough to effectively differentiate between positive and negative raw milk samples. To address this issue, the researchers gradually increased the amount of the phenol red indicator to enhance the visibility of colours. The results indicated that when 0.08g of phenol red was used, clearer distinctions were observed. Consequently, this amount was chosen for subsequent experiments.

In the second experiment, the researchers decided to eliminate one ingredient at a time from the reagents to investigate its impact on colour differences. However, the elimination of

any single ingredient did not yield significant distinctions in colour for all the experiments. Nonetheless, it was suspected that one particular ingredient might play a more significant role in contributing to the colour differences between positive and negative samples. Although the specific ingredient responsible was unknown, it became evident that the presence of all ingredients was necessary for the hydrolysis reaction to occur.

Moving on to the third experiment, the researchers decided to increase the quantity of each ingredient to three times the specified amount of HDBT. Among the reagents tested, reagent 7, which contained a higher concentration of trisodium phosphate, showed clear and distinct colour differences between positive and negative milk samples (as depicted in figure 3). On the other hand, reagents 6, 8, 9, and 10 exhibited insignificant differences in colour between positive and negative samples. To further evaluate the results, sensory evaluation was conducted, and the sample sets using reagent 7 were ranked as the best.

Subsequent experiments aimed to determine the optimal amount of trisodium phosphate required. Figure 4 illustrates the colour changes observed when trisodium phosphate was gradually added. Reagents 11, 12, 13, and 14, which contained 0.6g, 6g, 7.5g, and 9g of trisodium phosphate respectively, did not show significant colour differences. However, when 10.5g of trisodium phosphate was used in reagent 15, a better distinction between positive and negative beta-lactam samples was observed, similar to the results obtained with reagent 7 (figure 3). Notably, with reagent 16, which contained 12g of trisodium phosphate, the colour differences ceased.

The initial experiment lacked distinct colour differences, prompting the adjustment of the phenol red indicator. Subsequent experiments revealed the importance of all ingredients for the hydrolysis reaction, with trisodium phosphate playing a crucial role. Increasing the concentration of trisodium phosphate in reagent 7 resulted in clear colour distinctions between positive and negative samples. Further experimentation led to the determination of the optimal amount of trisodium phosphate required. The results highlighted the significance of precise ingredient composition in achieving accurate differentiation between positive and negative milk samples.

Figure 3: Colour observations when each ingredient amount was three times more of the HDBT reagent

Figure 4: Colour differences with gradual addition of trisodium phosphate

According to the results presented in figure 4, it is evident that reagent number 15 exhibited the most effective colour distinction, having the highest mean score and ranking as the best with a score of 300 points. The line scale method also demonstrated similar outcomes, showing that reagent number 15 had the highest mean score and was significantly different ($P \leq 0.05$) from the other reagents (as shown in table 6). Reagents number 13 and 12 did not exhibit significant differences ($P \leq 0.05$) from each other, similar to reagents number 12 and 11. Reagent number 14 showed significant differences from reagents 11, 12, 13, 15, and 16, but it did not rank as the best, and its mean score was over four times lower than that of reagent 15. Results from reagent number 16 (figure 4) indicated no distinction between positive and negative milk samples, displaying the lowest mean score and being significantly different ($P \leq 0.05$) from the other reagents. The colour differences between positive and negative samples were indistinguishable for reagent number 16.

Based on these findings, reagent number 15 was selected as the optimal choice and its composition was established as the recommended formula for the novel test method to

differentiate between positive and negative raw milk samples for beta-lactam antibiotic residues detection. The experiment concluded with the determination of the new reagent's ingredient composition, which is outlined in table 7. The main differences between the developed reagent and the original HDBT reagent lie in the quantities of trisodium phosphate and phenol red indicator used.

Table 6: Means for the various reagents having different amounts of phosphate

Reagents	Line scale method
11	2.10^{d}±0.27
12	2.31^{cd}±0.21
13	2.75c±0.26
14	3.43^{b}±0.38
15	14.26^{a}±0.10
16	0.34^{d}±0.04

Means within a column marked with different letters are significantly different at ($P \leq 0.05$)

Table 7: The composition of the modified test reagent

S/no.	Ingredient	Amount (g)/L of H_2O
1.	Penicillin	15.0
2.	Sodium Chloride	5.0
3.	Trisodium Citric Acid	1.5
4.	Trisodium Phosphate	10.48
5.	Phenol Red	0.08

4.1.2 Determination of Reagent: Raw Milk Mixing Ratio

Among the nine suggested mixing ratios of milk to reagent, it was observed that these three ratios, namely 4:6, 5:5, and 6:4, exhibited some distinction between a beta-lactam positive and a negative raw milk sample, unlike the other ratios. To determine the best ratio among these three, further analysis was conducted. Figure 5 below displays a photograph of the results obtained for the three ratios, as described. Each set of samples consists of a beta-lactam antibiotic residues positive milk sample in the left test tube and a beta-lactam antibiotic residues negative milk sample in the right test tube.

Figure 5: The results of the three mixing ratios

The panelists were instructed to utilize both the line scale and ranking methods to determine the best ratio among the three options. Based on their evaluations, ratio 5:5 (or otherwise 1:1) was ranked as the best, followed by ratio 4:6, while ratio 6:4 was ranked last. The results from the line scale method, as presented in Table 8, support this ranking. Ratio 5:5 exhibited the highest mean score and was significantly different ($P \leq 0.05$) from ratios 4:6 and 6:4. On the other hand, ratios 4:6 and 6:4 did not show a significant difference ($P \leq 0.05$) between them.

These findings indicate that, for this test method to achieve optimal performance, an equal proportion of milk and reagent should be mixed. This suggests that a 1:1 ratio, or specifically a 5:5 ratio, is recommended for efficient and accurate results in distinguishing between beta-lactam antibiotic residues positive and negative raw milk samples.

Table 8: Means for different milk and reagent mixing ratio

Ratio	Means
4:6	6.94^{b}±0.87
5:5	9.99^{a}±0.72
6:4	6.52^{b}±0.78

Means within a column marked with different letters are significantly different at ($P \leq 0.05$).

4.1.3 Determination of Type of Breeds Effect on the Test Method Results

In this test, both beta-lactam antibiotic residues positive and negative milk samples were used for the different sample sets. Figure 6, displayed below, presents the analysis of test results specifically for beta-lactam antibiotic residues positive raw milk samples obtained

from two different breeds. Each set of samples consisted of a Friesian milk sample on the left and an Ayrshire milk sample on the right. However, the panelists reported no discernible colour differences among any of the samples, making it impossible to rank them. Consequently, the mean results obtained from the line scale method indicated no significant difference ($P \leq 0.05$) between the results obtained from Friesian and Ayrshire milk samples. Table 9 provides the means for the six sets of samples, further supporting the lack of significant differences between the two breeds.

Table 9: Means for different sample sets having Friesians and Ayrshire milk samples

Set code	ABC	BCA	CBA	BBI	BAB	ABB
Means	$0.92^{a}\pm0.18$	$1.35^{a}\pm024$	$1.29^{a}\pm0.28$	$0.95^{a}\pm0.17$	$1.17^{a}\pm0.25$	$0.98^{a}\pm0.24$

Means within a row marked with different letters are significantly different at ($P \leq 0.05$)

Figure 6: Different set of Beta-lactam positive samples using the same reagent but milk samples from the different breeds

4.2 Determination of the Reagent's Shelf Life

Following the storage of the reagent under four different temperature conditions (frozen, refrigeration, room temperature, and under direct sunlight), it was observed that the viability of the reagent expired after the second day for all conditions. This was confirmed by the failure of all stored reagents to exhibit colour differences between beta-lactam positive and negative raw milk samples on the second day of analysis. Table 10 below presents the mean comparisons, indicating that the freshly prepared reagent was significantly different ($P \leq 0.05$) from all other reagents stored under different conditions. This outcome prompted an investigation into the reagent's shelf life in terms of hours, revealing that the reagent could be preserved for a maximum of 5 hours.

Efforts were made to find better ways to improve the reagent's shelf life. Initially, the ingredients listed in table 7 were used in powdered form. Through sensory evaluation, it was determined that the colours obtained when using the powder showed no significant difference ($P \leq 0.05$) compared to the colours obtained when using the reagent for both beta-lactam positive and negative raw milk samples (as shown in table 11 below). Moreover, the results indicated that using 0.05g of the powder in 3mls of milk resulted in more definite colours between beta-lactam antibiotic residues positive and negative raw milk samples. Figure 7 below showcase the observed colours when the powder was used. Notably, it became possible to differentiate between a positive and negative sample. In figure 7, the test tube on the left represents a negative sample, while the test tube on the right represents a positive sample.

Figure 7: Colour observations using ingredients as powder on a Beta-lactams positive (right test tube) and a negative (left test tube) raw milk sample

To improve the shelf life and facilitate the applicability of the test method, the powder was packed in ampoules under semi-vacuum conditions. Its shelf life was then assessed over a period of four months, initially on a weekly basis and later on a monthly basis. The investigation confirmed that the powder's keeping quality could surpass the four-month period, indicating its extended shelf life. Based on these findings, it is recommended to use the powder instead of the reagent. By utilizing the powder form, the shelf life of the test method can be enhanced, ensuring its usability and practicality for an extended duration. This allows for greater convenience and reliability in conducting the test and also enhances applicability of the method in the farm settings.

Table 10: Means comparisons for the freshly prepared and the stored reagents

Reagent source	Means
Fresh reagent	$13.94^{a}\pm0.13$
Frozen conditions	$0.4^{b}\pm0.01$
Refrigerated	$0.3^{b}\pm0.01$
Room temperature	$0.2^{b}\pm0.01$
Under sunlight	$0.1^{b}\pm0.01$

Means within a column marked different letters are significantly different at ($P \leq 0.05$)

Table 11: Means comparisons for the reagent and powder results

Test method	Means
Reagent	$0.183^{a}\pm0.01$
Powder	$0.181^{a}\pm0.01$

Means within a column marked different letters are significantly different at ($P \leq 0.05$)

4.3 Sensitivity and Specificity of Rapid Beta-Lactam Antibiotic Residues Detection Kits

The results presented in table 12 provide information on the sensitivity and specificity of all the five beta-lactam antibiotic residues test methods. The developed test method, known as the NAR test, exhibited a sensitivity of 66.7% and a specificity of 100%. On the other hand, the Mtusbio, Ringbio, Delvo BL, and Delvo SPNT tests demonstrated a sensitivity and specificity of 100%. These results indicate that the four alternative test methods are more sensitive in detecting beta-lactam residues compared to the NAR test.

The NAR test method successfully detected 10 samples as negative (antibiotic-free) and 10 samples as beta-lactam antibiotic residues positive. However, it failed to detect the presence of residues in 6 samples that were spiked with beta-lactam antibiotic residues positive milk. Additionally, 2 samples indicated partial positivity, where there was a mixture of positive and negative milk at ratios of 1:1 and 3:1, respectively.

The specificity of the NAR test method was 100%, indicating consistent and reproducible results across replications. The positive predictive value represents the accuracy of the tests in identifying truly positive samples with beta-lactam residues, while the negative predictive value reflects the accuracy in identifying truly negative samples that are antibiotic-free. The positive predictive value for the NAR test was 100%, while the negative predictive value was 62.5%.

The odds ratio (table 12) for the NAR test was 9.60, whereas for all the other tests, it was 195.37. Since the odds ratio was greater than one for all the test methods, there is a positive association among the test methods with the NAR test method. This implies that the NAR method had a 9.60 times higher likelihood of providing an accurate result compared to the other methods, which had a likelihood of 195.37.

Overall, the sensitivity, specificity, positive predictive value, negative predictive value (table 12), and odds ratio provide insights into the performance and accuracy of the different test methods for detecting beta-lactam residues.

Table 12: Percentage Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and the odds ratio of the NAR test in comparison with other tests

Test	Sensitivity	Specificity	PPV	NPV	Odds ratio
Delvo (BL and SPNT)/ Mtusbio/ Ringbio	100	100	100	100	195.37
NAR	66.7	100	100	62.5	9.60

Table 13: Kappa coefficient analysis results

Agreement	Expected agreement	Kappa	Standard error	Z	Probability
0.7857	0.4912	0.5882	0.1764	3.00	0.0014

According to the information provided in table 13, if both the NAR test method and any of the other methods made their determinations randomly, we would expect them to agree on approximately 49.12% of the test samples. However, in this case, they agreed on 78.57% of the samples. The higher level of agreement suggests that we can reject the hypothesis that the methods are making their determinations randomly.

The Kappa coefficient, calculated as 0.5882 (table 13), falls within the range of 0.40 to 0.60 on the Landis-Koch scale. This indicates a moderate agreement between the NAR test method and the other test methods. On the other hand, for all the other tests, there was 100% agreement as they were able to detect all test samples as expected. Based on the analysis, it can be concluded that there was a significant agreement ($P \leq 0.05$) between the NAR test method and the other test methods, indicating that they are not making their determinations randomly and that there is consistency in their results.

CHAPTER FIVE
DISCUSION

5.1 General overview on antibiotic residues

Even when antibiotics are used judiciously, they have been associated with triggering allergic reactions such as urticaria and bronchoconstriction, as well as severe conditions like immune-mediated hemolytic anemia and intravascular hemolysis in animals and humans (Maker *et al.,* 2019). Moreover, certain beta-lactam antibiotics have been found to exhibit neurotoxic, nephrotoxic, genotoxic, and urogenital toxicity effects in humans (Maker *et al.,* 2019). Antibiotics are generally recommended to be administered for a minimum of five consecutive days with the same daily dose to ensure effective treatment in both humans and cattle. However, the pursuit of profit-making and cost reduction in the cattle industry often leads to non-compliance with treatment protocols. Farmers and veterinarians frequently opt for shorter treatment schemes, not only to avoid interrupting milk production but also to save costs associated with medication and general cattle care.

While the majority of research studies on antibiotic residues have been performed in Europe (46.88%) followed by Asia (34.38%), South America (8.04%), North America (7.14%), and Africa (3.57%) (Sachi *et al.,* 2019), recent literature from Kenya provides insights into the presence of antibiotic residues in milk samples within the country. A study by Mitema *et al.* (2016) examined smallholder dairy farms in Nakuru County and found a significant prevalence of antibiotic residues, including beta-lactams, in 55% of the analyzed milk samples. This highlights the need for improved antibiotic use practices and effective monitoring systems to ensure milk safety and protect public health.

Another study conducted by Onono *et al.* (2019) investigated antibiotic residues in milk from urban and peri-urban dairy farms in Nairobi County, Kenya. The findings of this study revealed the presence of antibiotic residues, including beta-lactams, in 35% of the analyzed milk samples. The authors emphasized the importance of raising awareness among dairy farmers about responsible antibiotic use and establishing robust residue monitoring programs.

Similarly, a study by Mburu *et al.* (2021) focused on smallholder dairy farms in Kiambu County, Kenya. The research assessed antibiotic use practices and the presence of antibiotic residues in milk samples. The study found that 40% of the milk samples tested positive for antibiotic residues, including beta-lactams. These findings highlight the need for promoting proper antibiotic use, adherence to withdrawal periods, and effective monitoring to ensure milk safety and mitigate the risks associated with residues.

These recent studies conducted in Kenya demonstrate that antibiotic residues, including beta-lactams, are prevalent in a significant proportion of milk samples from various regions of the country. They underscore the importance of promoting responsible antibiotic use, increasing awareness among farmers, and implementing robust monitoring systems to ensure milk safety and minimize the potential health risks posed by antibiotic residues.

In conclusion, the presence of antibiotic residues in milk poses risks to both animal and human health. While the majority of research studies on antibiotic residues have been conducted in Europe and other regions, recent literature from Kenya highlights the need for further research and targeted interventions to ensure responsible antibiotic use, minimize residues, and safeguard public health in the country. This informed the basis of this study.

5.2 Modification of HDBT ingredients in test method development (Objective 1)

Beta-lactam antibiotics are a class of antimicrobial agents that contain a beta-lactam ring in their chemical structure. These antibiotics exert their therapeutic effects by inhibiting the biosynthesis of bacterial cell walls. However, bacterial resistance to beta-lactam antibiotics has become a significant concern. One of the primary mechanisms through which bacteria develop resistance is by producing beta-lactamase enzymes, which can degrade the beta-lactam ring and render the antibiotics ineffective (Pandey & Cascella, 2019). In addition to beta-lactamase production, other mechanisms such as reduced antibiotic penetration, alterations in target site penicillin binding proteins (PBPs), and active efflux mechanisms contribute to the overall resistance phenotype (Drawz & Bonomo, 2010).

To overcome beta-lactamase-mediated resistance, beta-lactam antibiotics are often co-administered with beta-lactamase inhibitors. One commonly used inhibitor is clavulanic acid, which acts as a mechanism-based beta-lactamase inhibitor (Pandey & Cascella, 2019). By binding irreversibly to the active site of beta-lactamases, clavulanic acid prevents the degradation of beta-lactam antibiotics, thus restoring their bactericidal activity (Drawz & Bonomo, 2010). This combination therapy has been effective in combating resistance in bacterial strains that produce beta-lactamase enzymes (Pandey & Cascella, 2019).

Beta-lactamases, also known as penicillinases, are enzymes classified under the Enzyme Commission (EC) number 3.5.2.6. As mentioned previously, beta-lactamases are divided into four categories (class A, B, C, and D) based on their amino acid sequence. Several researchers have reported that different β-lactamases may share a certain degree of homology. They are capable of hydrolyzing the beta-lactam ring present in a wide range of beta-lactam antibiotics, including penicillins, cephalosporins, monobactams, and

carbapenems (Shaheen *et al.,* 2013). The hydrolysis of the beta-lactam ring occurs through the action of a serine residue in the active site of the enzyme, resulting in the formation of a covalent acyl ester and the inactivation of the antibiotic (Bonomo, 2017). However, certain beta-lactamases, known as class B enzymes, utilize a zinc ion instead of a serine residue to attack the beta-lactam ring (Bonomo, 2017).

The detection of penicilloic acid, a product of beta-lactam ring hydrolysis, has been used as an indicator of beta-lactam antibiotic residues. Li *et al.* (2014) conducted a study using ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry to investigate the degradation of penicillin in milk by beta-lactamase enzymes. They found that the enzymatic degradation resulted in the formation of penicilloic acid and penilloic acid. Similarly, Shaheen (2013) reported that beta-lactamases hydrolyze the amide bond in the beta-lactam ring, leading to the production of penicilloic acid. Harris (2015) also confirmed that the hydrolysis of the beta-lactam ring by penicillinase enzymes yields penicilloic acid, which lacks bactericidal activity. Furthermore, Canzani and Aldeek (2017) investigated the stability of penicillin G and identified penicilloic acid, penillic acid, and penilloic acid as the major metabolites. They developed an iodometric method based on chromophore decolourization for the determination of penicilloic acids.

The Hardy Diagnostics Beta-Lactamase Test (HDBT) is an acidometric method commonly used to detect beta-lactamase production by various bacterial species, including *Neisseria gonorrhoeae, Haemophilus,* and *Staphylococcus*. The HDBT test relies on the hydrolysis of penicillin by beta-lactamase enzymes produced by the bacteria. This hydrolysis causes a drop in pH, leading to a colour change in the phenol red indicator from fuchsia purple to yellow (Hardy Diagnostic Catalogue, 2018). The presence of beta-lactamase enzymes in raw milk samples can be exploited in the HDBT test for the detection of beta-lactam residues. If beta-lactamases are present, hydrolysis of the penicillin ring occurs, resulting in the production of penicilloic acid and a subsequent colour change (Li *et al.,* 2014).). In another study, when phenol red indicator was mixed with various carbohydrates, it makes mediums that can assist in differentiating various bacterial species that can ferment specific carbohydrates producing acid. The production of the acid exhibits a change from Fuschia Purple to yellow (Kali *et al.,* 2015). The broth of phenol red has also been used as a differential test medium in gram negative enteric bacteria. If the organism is able to utilize the carbohydrate, an acid by-product is created, which turns the media yellow (Raffy *et al.*, 2022). Similarly, phenol red has been used in detection of other enzymes. In their study, Surre *et al.* (2018) on enhanced detection of carbapenemase-producing Enterobacteriaceae by

an optimized phenol red assay, they were able detect production of the enzyme based on the colour and its profile change. They termed their test as simple and robust. Use of phenol red in detecting other enzymes has also been evaluated. Carbapenemase enzyme test based on visual observations and pH change was also suggested by Nordmann *et al.* (2012). In their study to evaluate production of the enzyme, a test reagent comprised of the carbapenemase enzyme, bacterial cells and the phenol red pH indicator. For bacteria cells that produced the enzyme, the medium changed from fuchsia purple to yellow. According to Tamma and Simmer (2018), in the presence of the carbapenemase enzyme producing strain, when hydrolysis of the carbapenem beta-lactam ring occurs, a carboxyl group results consequently leading to a drop in pH in the culture medium. Consequently, the pH indicator shifts from red to yellow, indicating the presence of a bacteria producing carbapenemase enzyme.

In conclusion, beta-lactam antibiotics face challenges due to bacterial resistance mechanisms, including the production of beta-lactamase enzymes. Combination therapy with beta-lactamase inhibitors, such as clavulanic acid, has proven effective in overcoming resistance. The detection of penicilloic acid and the HDBT test provide valuable tools for identifying beta-lactam residues in milk samples. These methods contribute to ensuring the safety and quality of dairy products and monitoring the appropriate use of beta-lactam antibiotics in the livestock industry.

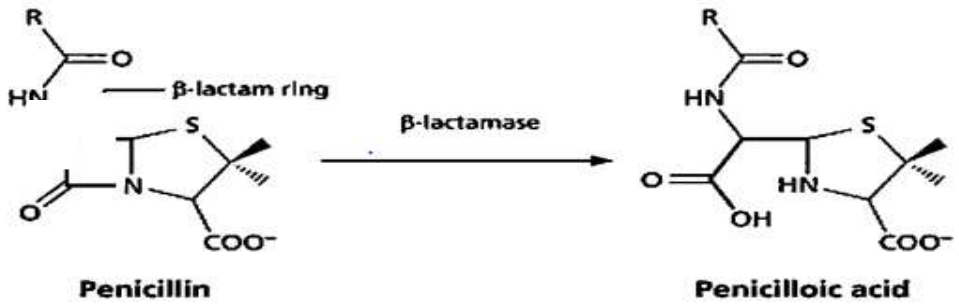


Figure 8: Hydrolysis of Beta-lactam antibiotics by Beta-lactamase enzymes (Harris, 2015)

In this modified test method, the HDBT reagent was altered by increasing the quantity of trisodium phosphate and phenol red indicator to improve the results, leading to distinct colour changes. This adjustment allowed for better differentiation between beta-lactam antibiotic residues positive and negative raw milk samples. Trisodium phosphate and trisodium citrate are components of the Mcllvaine buffer, which is used in colourimetric comparisons and prepared within a pH range of 2.2 to 8.0. By increasing the amount of trisodium phosphate, which is a strong alkali, the pH of the solution tends towards alkaline

conditions, creating an optimal environment for the reaction (Mcllvaine, 1921). A buffer solution should provide the appropriate medium in which reactions will occur. The main function of a buffer is to resist changes in pH when adding strong acids or bases (Brooke *et al.* 2015). Buffered system also provides cofactors for enzymatic reactions, critical salts and even essential nutrients for cells and tissues (Bisswanger, 2014).

Trisodium phosphate (TSP) is an inorganic compound represented by the chemical formula Na_3PO_4. It is a white, granular or crystalline solid that readily dissolves in water, producing an alkaline solution. TSP is commonly utilized as a cleaning agent, builder, lubricant, food additive, stain remover, and degreaser (Tognonvi *et al.*, 2012). Trisodium phosphate was used as a measure to enhance cleaning of dairy equipment as well as bactericidal action. This was because of its alkaline properties, which is approximately pH 11 (Sarjit & Dykes, 2017). The alkaline property made trisodium phosphate a valuable ingredient in the NAR test method as it enabled the distinction between a positive and negative test sample.

Phenol red indicator, also known as phenolsulfonphthalein, is a pH-sensitive dye that exhibits a gradual colour transition from yellow to red within a pH range of 6.2 to 8.2. Beyond a pH of 8.2, the dye appears as a bright fuchsia colour (Held, 2018). Phenol red is water-soluble due to its polarity and strong hydrogen bonding capabilities (Cuypers, 2010). In a solution with low pH, it carries a negatively charged sulfate group and a positively charged ketone group. As the pH increases (pKa=1.2), the excess proton in the ketone group is released, resulting in a yellow colour. Therefore, when penicilloic acid is produced following the hydrolysis of the beta-lactam ring, the pH decreases, causing phenol red to change from fuchsia purple to peach.

Li *et al.* (2014) demonstrated that penicillin decomposes into penicilloic acid in an aqueous solution through the action of either an alkali or bacterial enzymes. This could explain the improved colour observation when the concentration of trisodium phosphate was increased, as the medium became more basic. As the quantity of trisodium phosphate continued to increase, the pH of the medium became more alkaline. When the pH exceeds 8.2, phenol red changes to a bright fuchsia purple, making it difficult to distinguish between positive and negative raw milk samples. According to Held (2018), further increases in pH (pKa=7.7) result in the loss of hydrogen from the hydroxyl group, causing the colour to change to red. The combination of the sulfate group, ketone group, and hydroxyl group in phenol red enables it to function as a pH indicator dye. As demonstrated in this study, an optimal pH condition for the enzyme and indicator was achieved with a mixing ratio of 1:1,

leading to favorable outcomes. Li *et al.* (2014) identified beta-lactamase dosage and pH as the major determinants in the hydrolysis reaction. Increasing the pH from 2 to 6 in the presence of beta-lactamase enzymes resulted in an enhanced response in penicilloic acid production. Additionally, higher quantities of beta-lactamase enzymes increased the degradation of penicillin.

Several other studies have employed different methods to detect beta-lactams or antibiotic residues. For example, Ibrahim *et al.* (2013) conducted a study on the detection and risk assessment of beta-lactam residues in Kosovo's milk using the ELISA method. Out of 50 analyzed milk samples, 6 were found to be contaminated with beta-lactam residues. However, the sensitivity of the method used was questioned, as more than ten samples were expected to contain beta-lactams residues. In another study conducted by Alkan (2008) in Turkey, the confirmation of commercial kits used for detecting antibiotics in milk was performed using HPLC. Out of 81 milk samples analyzed, only two were found to be contaminated with beta-lactam residues, whereas other methods predicted that more than fifteen samples would contain the same residues. These studies concluded that a more suitable method was required to improve the detection of residue levels.

Nordmann *et al.* (2012) developed a similar test, based on the detection of beta-lactamase enzymes as an indicator of beta-lactams antibiotic residues using phenol red. They devised a test for the rapid identification of Extended-Spectrum-Beta-Lactamase (ESBLs) in *Enterobacteriaceae.* This test relied on the detection of beta-lactam (cephalosporin) hydrolysis, which could be reversed by adding tazobactam. The activity of ESBLs was indicated by a colour change from red to yellow, observed through phenol red indicator. The findings of their experiment align with the principles used in the NAR method study. Furthermore, the presence of penicilloic acid has been utilized to detect the presence of penicillin in milk. For instance, Liu *et al.* (2011) developed a rapid, sensitive, and specific method for determining penicillin G, benzyl penicilloic acid, benzyl penilloic acid, and benzyl penillic acid in bovine milk using ultra-high-performance liquid chromatography–tandem mass spectrometry. Their established method was successfully applied to detect penicillin and its major metabolites in bovine milk samples. The primary metabolite identified was penicilloic acid, which was found in 20% of the bovine milk samples at an average concentration of 320 ng/mL. Gaare *et al.* (2012) stated that the "novel beta-lactamase induction-based principle can be applied for specific detection of beta-lactam antibiotic residues in dairy foods." They successfully detected beta-lactam antibiotics in milk using the indicator strain Bacillus cereus, which produces beta-lactamase enzymes upon induction. The

test ampoules containing spores were tested for induction using penicillin G in spiked milk, and their findings exhibited complete correlation with other reference methods.

Differences exists between Friesian and Ayrshire breeds and several factors and variables have been associated. The changes in composition can be brought about by many factors including breed and genotype, nutrition and foraging system, season, parity, stage of lactation as well as the physiological state of the animal (Hanuš *et al.* 2018). Previous studies have established the potential to exploit variation of milk composition among breeds to improve on milk quality. Citrate for example plays a role in milk processing by interacting with other milk constituents to influence the coagulation of milk protein and produces the aromatic flavour of fermented milk product. Citrate content is known to be higher in Jersey and Ayrshires cows than in Holstein Friesians. Based on these findings, there was a need to investigate the breed effect in the developed test results. The investigation did not show significance difference between the breed in the NAR test results. This could be because the efficiency of the test depends on the presence of the beta-lactamase enzyme in the milk sample. In addition, studies had shown that the enzyme exists endogenically in raw milk following treatment with beta-lactam antibiotic drug residues irrespective of the breed. In addition, and because several types of proteins are present in milk, it is crucial to assess any cross-reactivity in milk using the developed beta-lactamase detection method to assess its efficiency.

5.3 Determination of the Reagent's Shelf Life (Objective 2)

The stability of the reagent used in the test was compromised when stored under various conditions, including direct sunlight, room temperature, refrigeration, and freezing. Regardless of the storage conditions, the reagent proved to be unstable even for a short period, posing challenges for its practical use in field conditions. Consequently, the shelf life of the reagent was investigated and determined to be only 5 hours. Further investigations focused on the individual ingredients of the reagent, without the addition of distilled water. These investigations revealed that the shelf life of the reagent significantly differed from that of the powder. However, the colour distinction achieved when using both the reagent and the powder was not significantly different. This lack of distinction could be attributed to the hydrolysis of the phenol red indicator by water, rendering it ineffective in differentiating between positive and negative milk samples. Wade (2018) explains that phenol undergoes slow oxidation when exposed to air and water, resulting in the formation of a quinone that reacts with phenol to form a phenoquinone. This phenomenon may explain why packaging

the powder under semi-vacuum conditions, which prevents the absorption of atmospheric water, prolongs its shelf life. Moreover, storing the reagent under direct sunlight did not yield significantly different results, as the chromophoric nature of phenol red leads to light absorption and reduced effective illumination in the near-UV range (Enderle, 2012).

Another factor that may have influenced the shelf life of the reagent is the chemical reaction between trisodium phosphate and water. When these two substances react, they form a solution with sodium and phosphate ions as the resulting products (Wade, 2018). The positive ionic charge of sodium ions and the three negative ionic charge of phosphate ions may have contributed to the reagent's inability to detect the difference between a beta-lactam positive milk sample and a negative sample. The presence of distilled water, which contains oxygen, could have enhanced the reactivity of the reagents. Consequently, further research is warranted to validate these findings and investigate the specific reactions and interactions between the reagent ingredients and water.

5.4 Sensitivity and Specificity of the Test Method in Comparison with Other Rapid Beta-Lactam Antibiotic Residues Detection Kits (Objective 3)

There was a necessity to compare the newly developed NAR test with other rapid kits available in the market to assess its performance and applicability in comparison. The NAR test method demonstrated a sensitivity of 66.7% and a specificity of 100%, indicating its ability to accurately identify positive and negative samples, respectively (Ndungu *et al.,* 2021). However, the sensitivity of the NAR test was lower for the six milk samples spiked with known residues, with two samples showing partial positivity. This reduction in sensitivity could be attributed to the lower concentration of beta-lactam residues in the spiked milk samples. On the other hand, the other four test methods exhibited 100% sensitivity, detecting all positive samples correctly.

Despite the lower sensitivity for spiked samples, the NAR test method successfully identified all negative (antibiotic-free) samples and all beta-lactam positive samples, resulting in a specificity of 100%. This indicates the reproducibility of the test, as it consistently provided the same results for replicates (Ndungu *et al.,* 2021). The positive predictive value (PPV) of the NAR test was 100%, indicating that all positive results were indeed accurate, while the negative predictive value (NPV) was 62.5%, indicating that there was a possibility of false negatives (Ndungu *et al.,* 2021). In contrast, the other test methods achieved a PPV and NPV of 100% for both categories.

To improve the sensitivity and NPV of the NAR test, further research is needed, including the development of quantitative methods (Ndungu *et al.*, 2021). However, considering that the NAR test is intended for use at the farm level, prior to bulk collection of milk from individual farmers, its accuracy in detecting residues is still valuable. This quality control system allows for immediate acceptance or rejection of milk on-site, with the option for further testing if discrepancies occur.

The Kappa coefficient, which measures the agreement between different test methods, indicated moderate agreement between the NAR test method and the other methods (Ndungu *et al.,* 2021). According to the Landis-Koch scale, this suggests that the NAR test method is reasonably applicable and reliable. The odds ratio for the NAR test method was 9.60, while for the other test methods, it was 195.37 (Ndungu *et al.*, 2016). As the odds ratio was greater than 1 for all test methods, there is a positive association among the test methods, particularly for samples known to have residues and samples known to be residue-free.

In the comparative analysis, all antibiotic-free milk samples tested negative, while all milk samples with beta-lactam residues tested positive for all four test methods. These findings provide evidence that these rapid screening tests, including the NAR test method, are potentially useful tools for monitoring raw milk products for the presence of antibiotic residues (Ndungu *et al*., 2021).

The NAR test method's sensitivity is dependent on the presence or absence of the beta-lactamase enzyme in raw milk, as its availability determines the detectability of residues. However, the test may not be able to identify antibiotics intentionally added to milk as adulterants, as the beta-lactamase enzyme would not be formed in such cases. Dilution effects, as observed when raw milk is bulked in a cooling tank, can also influence the test's sensitivity. Despite these limitations, the NAR test method accurately detects both negative and positive samples, making it suitable for on-farm or milk collection route testing before bulk transportation (Ndungu *et al.,* 2021). Several studies have associated the concentration of enzymes as one of the factors affecting their visual detection. In a study carried out by Surre *et al*. (2018), the more concentrated the carbapenemase enzyme in the samples the better the detection test results. This is because an increase in substrate concentration leads to an increase in the rate of an enzyme-catalyzed reaction and their activity can often be detected only at their optimum conditions. When milk is pasteurized, the concentration of the enzyme decreases although it cannot be fully eliminated (Shuang *et al.,* 2015).

In terms of cost, analyzing antibiotic residues becomes a significant factor when dealing with a large number of samples, such as in the Kenyan smallholder supply chain with

over 1.8 million dairy farmers (KDB, 2017). Therefore, it is crucial to develop cost-effective methods to ensure economic sustainability (Layada *et al.*, 2016). The existing rapid antibiotic residues kits in Kenya have been deemed expensive and unsustainable (Mwagore *et al.*, 2019). Hence, there is a need to develop cheaper test methods to facilitate widespread testing of raw milk for residues. Furthermore, concerns have been raised regarding the high occurrence of false-positive results with on-farm rapid antibiotic screening tests (Mullen *et al.*, 2017), underscoring the importance of implementing quality control testing systems in raw milk collection chains to ensure food safety.

The USA has implemented antibiotic stewardship programs in healthcare facilities and regulations in animal agriculture to promote responsible antibiotic use. These efforts aim to reduce the economic burden associated with antibiotic resistance, which is estimated to cost the US healthcare system billions of dollars annually (O'Neill, 2016). In Europe, the economic impact of antibiotic resistance is also substantial, with estimated costs ranging from billions to tens of billions of Euros per year (Kastrinos and Weber, 2020). The EU has implemented the One Health approach, focusing on surveillance, prevention, and research to address antibiotic resistance comprehensively. This approach aims to minimize economic losses in healthcare, agriculture, and other sectors. The Netherlands has been particularly successful in reducing antibiotic consumption and resistance levels. Their "One Health" approach, coupled with strict regulations and targeted interventions, has led to significant economic benefits. It is estimated that the Netherlands saves approximately 21 to 48 million Euros per year due to reduced healthcare costs associated with antibiotic-resistant infections (Huijbers *et al.*, 2019).

In Kenya, the economic losses associated with antibiotic resistance are also significant. Limited access to effective antibiotics, coupled with the high prevalence of substandard and counterfeit drugs, exacerbates the economic impact (O'Neill, 2020). However, Kenya is working towards strengthening its antibiotic stewardship programs and surveillance systems to address these challenges and reduce economic losses. It is essential for Kenya to address the economic losses associated with antibiotic resistance by investing in surveillance, promoting responsible use, and ensuring access to quality-assured antibiotics.

Consumer awareness initiatives play a crucial role in addressing the issue of antibiotic residues in the milk value chain in Kenya. These initiatives aim to educate consumers about the potential health risks associated with consuming milk contaminated with antibiotic residues. By raising awareness, consumers can make informed choices and demand milk that meets safety standards. However, there are challenges in detecting antibiotic residues in the

milk value chain, including limited resources, inadequate testing facilities, and the presence of substandard and counterfeit drugs in the market (Layada *et al.*, 2016).

In Kenya, the detection of antibiotic residues in the milk value chain poses significant challenges. Limited resources, both in terms of equipment and trained personnel, hinder the implementation of comprehensive testing programs. Additionally, the presence of substandard and counterfeit drugs in the market makes it difficult to ensure the quality and efficacy of antibiotics used in dairy farming (Mwagore *et al.,* 2019). These factors contribute to the complexity of detecting antibiotic residues in milk samples and enforcing safety standards.

Efforts have been made to improve the detection of antibiotic residues in the milk value chain in Kenya. For example, the Kenya Dairy Board (KDB) has implemented regulations and guidelines to ensure the safety and quality of milk products. However, effective implementation and enforcement of these regulations remain a challenge due to limited resources and capacity (Mwagore *et al.,* 2019). To address these challenges, there is a need for increased investment in testing facilities, training programs for dairy farmers and milk processors, and collaboration between stakeholders in the milk value chain (Ndung'u *et al.*, 2016). Also, innovation of cheaper methods for detection such as the one present in this study will be an added advantage.

5.5 Cost Analysis of the Reagents

The development of a new method for beta lactams determination using a cost-effective approach aligns with Kenya's commitment to the Sustainable Development Goals (SDGs) and its national development agenda, Vision 2030 (United Nations, 2016; Republic of Kenya, 2008). The SDGs, adopted by Kenya and other United Nations member states in 2015, provide a framework for sustainable development and address various social, economic, and environmental challenges (United Nations, 2016).

In particular, this method contributes to SDG 3, which aims to ensure healthy lives and promote well-being for all. By accurately detecting milk adulteration through the detection of beta lactams, this method helps safeguard public health by ensuring the safety and quality of dairy products consumed by the population. It also supports SDG 2, which focuses on achieving food security and promoting sustainable agriculture, by helping to prevent fraudulent practices in the dairy industry that could compromise food safety.

Furthermore, the development of a cost-effective method aligns with Kenya's Vision 2030, a long-term development blueprint that aims to transform the country into a newly

industrializing, middle-income country. Vision 2030 emphasizes the importance of innovation, research, and development in driving economic growth and promoting sustainable development (Republic of Kenya, 2008). By reducing the cost of beta lactams determination, this method enhances affordability and accessibility, making it more feasible for widespread adoption within the dairy industry.

The cost analysis conducted for the production of the powder reveals that the raw materials required contribute to a total cost of 8,100 Kenyan shillings. These raw materials, such as penicillin and various reagents, are crucial for the formulation of the powder and its effectiveness in detecting milk adulteration. However, it is essential to consider that this cost estimate does not encompass other factors like labor, packaging, and innovation fees. These additional costs need to be factored in to obtain a comprehensive understanding of the total cost per test.

Considering the quantity of powder needed per single test, which is 0.05g in 3ml of milk, it is estimated that approximately 641 tests can be conducted using the total quantity of powder produced, approximately 32.1g. This estimation indicates the scalability and potential cost-effectiveness of the production process. Based solely on the raw material expenses, the approximate cost per test is calculated to be 25 Kenyan shillings. However, when considering all associated expenses including labor, packaging, and innovation fees, the cost per test is expected to increase by approximately ten Kenyan shillings.

In conclusion, the development of a cost-effective method for beta lactams determination not only addresses the SDGs, particularly SDG 3 and SDG 2, but also aligns with Kenya's Vision 2030. By ensuring the safety and quality of dairy products, promoting sustainable agriculture, and driving innovation, this method contributes to the overall socio-economic development of Kenya while addressing the global goals of sustainable development.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. This innovative color reaction test will play a pivotal role in enhancing milk acceptance procedures by differentiating milk that is suspected to contain residues and rejecting milk that has been conclusively determined to harbor residues. The proposed test methodology holds the potential to serve as an initial screening tool for identifying the presence of beta-lactam antibiotic residues right at the milk reception platform. This application can be extended throughout the milk collection process, effectively pinpointing residues prior to the consolidation of milk batches.

ii. From the study, the shelf-life of the test method when the ingredients are in form of a powder under semi-vacuum packaging stayed longer as compared to the freshly prepared test reagent. The results given by the powder and the freshly prepared test reagent were similar. However, more research needs to be done to understand the longevity of storage. The anticipated colors for a sample testing positive for beta-lactam antibiotic residues are expected to be peach or pink. Conversely, for samples devoid of beta-lactam antibiotic residues, the colors anticipated are fuchsia purple.

iii. From the study, the sensitivity and specificity of the NAR method as compared to the other testing methods was good and the method gave reliable results, indicating the potential of the utilization of the test method at the farm levels.

iv. This study verifies that beta-lactamase enzyme is present in raw milk following treatment with beta-lactam. Therefore, when the enzyme is detected, it should indicate presence of beta-lactam drug residues. This test method can appropriately be used by the farmers at the farm level for detection of the antibiotics residues in order to ensure antibiotic free milk is supplied.

6.2 Recommendations

i. As deduced from the results, the colour intensity difference between a positive and negative sample increased with increase in the level of the trisodium phosphate in the reagent. This can be used to differentiate between the presence and to some extent the quantity of the antibiotics hence having a capability to be used as a test method for detecting the antibiotics.

ii. Where the raw milk samples had higher amount of antibiotics residues, the colour strength was high tending towards being yellow. Meaning the yellow colour strength

increased with the level of antibiotics in raw milk samples hence a better preliminary test for antibiotics test at on-farm level due to simplicity in the colour differentiation.

iii. A vacuum packaging technology to package the test powder can be used to extend the shelf-life. This would ensure prolonged shelf life even at room temperatures. The size of the ampoules to be used in packaging the powder may be longer to hold the 6mls milk and preferably made of glass to facilitate visual observations.

6.3 Areas for Further Study

i. With the colour intensity change as the antibiotics' residues increase, a further study can be done using different light wavelengths to determine quantities of antibiotics.

ii. Further investigations should be carried out on the test method to evaluate the possibility of detecting other types of residues for instance tetracycline and sulfonamide using the same reagents or modification of the test method.

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APPENDICES

Appendix A: Antibiotic Residues Reported in Milk around the World

Antibiotic	No. of Samples	Milk category	Residues level (µg/L)	Country	Reference
Oxytetracycline	120	Pasteurized	150.4	Turkey	(Kaya and Filanzi, 2010)
Penicillin G	120	Pasteurized	33.5	Turkey	(Kaya and Filanzi, 2010)
Neomycin	120	Raw	7688.4	Turkey	(Kaya and Filanzi, 2010)
Penicillin G		Raw	59.53	Pakistan	(Khaskheli *et al.*, 2008)
Amoxicillin		Raw	36.11	Pakistan	(Khaskheli *et al.*, 2008)
Ampicillin		Raw	46.91	Pakistan	(Khaskheli *et al.*, 2008)
Amoxicillin			≤ 8		
Ampicillin			≤ 6		
Ceftioufur			≤ 20		
Cephapirin		Raw and Processed	≤ 12		
Penicillin	80		≤ 4	Kenya	(Kosgey *et al.*, 2018)
Sulfamethazine			≤ 10		
Tetracycline			≤ 50		
Chlortetracycline			≤ 100		
Oxytetracycline			≤ 50		
Gentamicin			≤ 30		

Appendix B: Level of contamination of milk with antibiotic residues around the world

Antibiotics	No. of samples	% Level of contamination	Country	References
penicillin G, gentamicin, oxytetracycline, neomycin and streptomycin	240	1.25%	Tukey	(Kaya and Filanzi, 2010)
ß – lactam	137	36.50%	Pakistan	(Khaskheli *et al.*, 2008)
penicillin G- type residues	1109	14.9%	Kenya	(Shitandi, 2001)
Beta-lactam, tetracyclines, macrolides, sulfonamides and aminoglycosides	986	36%	Tanzania	(Kurwijila *et al.*, 2006)
Beta-lactam, tetracyclines, and sulfonamides	480	24%	Kenya	(Ahlberg *et al.*, 2016)
Antimicrobials	206	21.1%	Kenya	(Wanjala *et al.*, 2018)
Amoxicillin, Ampicillin, Chlortetracycline, Ceftioufur, Cephapirin, Penicillin, Sulfamethazine, Tetracycline, Gentamicin, Oxytetracycline,	55	24%	Kenya	(Kosgey *et al.*, 2018)
36 veterinary drugs of penicillins, quinolones, macrolides, tetracyclines, sulfonamides, and trimethoprim	194	65.5 %	Algeria	(Layada *et al.*, 2016)
13 veterinary drugs of tetracyclines and sulphonamides	Rural: 229; Peri-urban: 80	Rural dairy system: 31.4%; Peri-urban dairy system: 28.8%	Kenya	(Orwa *et al.*, 2017)

Appendix C: Test Methods Commonly Used in Analysis of Antibiotic Residues in Milk

Class of antibiotic residue	Method	Reference
Penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin	TLC (Thin Layer Chromatography)/ Bioautographic method	(Kaya and Filanzi, 2010)
Beta-Lactam and sulfonamide	Delvotest SP test	(Yamaki *et al.*, 2004)
Beta-lactam	Microbial screening test (*bacillus subtilus* field disc assay) and high-performance liquid chromatography (HPLC)	(Khaskheli *et al.*, 2008)
Quinolones, sulphonamides, macrolides, anthelmintics and one tetracycline	Ultra-high-pressure liquid chromatography coupled to tandem quadrupole mass spectrometry	(Aguilera-Luiz *et al.*, 2008)
Five Beta-lactam and two cephalosporin	High-performance liquid chromatography with tandem mass spectrometry	(Holstege *et al.*, 2002)
BETA-lactam, tetracyclines, quinolones, amphenicols and sulfonamides	Capillary zone electrophoresis coupled with diode array detection	(Vera-Candioti *et al.*, 2010)
Five fluoroquinolone: norfloxacin, ciprofloxacin, ofloxacin, enrofloxacin, and rufloxacin.	High-performance liquid chromatographic (HPLC) method coupled with tandem mass spectrometry via electrospray ionization source (LC-MS/MS)	(Tang *et al.*, 2009)
Macrolides, aminoglycosides, cephalosporins, penicillins, quinolones, tetracyclines, sulphonamides, lincosamides, phenicolated and miscellaneous drugs	Microbiological method: five-plate test, called screening test for antibiotic residues (star)	(Gaudin *et al.*, 2004)
Streptomycin (SM), tetracycline (TC), and	Combine a multicolour quantum dot (QD)-based immunofluorescence assay	(Song *et al.*, 2015)

penicillin G (PC-G) in milk.	and an array analysis method	
Chloramphenicol, ampicillin, benzylpenicillin, dicloxacillin and erythromycin	High-performance thin-layer chromatography (HPTLC) combined with bioautography	(Ramírez *et al.*, 2003)
Sulfonamides and quinolones (sulfamonomethoxine, sulfadimethoxine, sulfamethazine, sulfamerazine, sulfaquinoxaline, enrofloxacin, and ciprofloxacin	Microbial assays and HPLC	(Chung *et al.*, 2009)
Penicillin G- type residues	Improved Dutch tube diffusion test	(Shitandi, 2001)
Beta-lactam, tetracyclines, aminoglycosides, macrolides, and sulfonamides	Charm-aim screening test kit	(Kurwijila *et al.*, 2006)
BETA-lactam antibiotic	Improved tube test	(Shitandi and Kihumbu, 2004a)
BETA-lactam, sulfonamides and tetracyclines.	Delvotest® screening test.	
BETA-lactam, sulfonamides and tetracyclines	Delvotest® screening test and trisensor test	(Ahlberg *et al.*, 2016)
Antimicrobials	Copan test kit with *Bacillus stearothermophyilus* var. *Calidolactis* spores, nutrients and a ph indicator (Bromocresol purple)	(Wanjala *et al.*, 2018)
Tetracyclines, sulfamethazine, Beta-lactam, and gentamicin	Idexx snap tests	(Kosgey *et al.*, 2018)
BETAeta lactamase	Sandwich ELISA	(Wang *et al.*, 2013)

Appendix D: Publication on Detection Kits for Beta-lactams Residues

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Full Length Research Paper

Evaluation of rapid Beta-lactam antibiotic residues detection kits for raw milk

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Platform tests serve as common and rapid quality control measures for assessing raw milk, aiding in deciding whether to accept, set aside, or reject it. Within the Kenyan market, various swift antibiotic residue test kits are available, such as Delvo test Fast BL, Delvo Sulphadiazine Penicillin No Tablet (SPNT), Mtusbio Betalactam BLQ Rapid Test Kit, and Ringbio Beta-lactam, tetracycline, sulfa drugs, BTS 3 in 1 TriTest S. In contrast, the Ndungu Antibiotic Residues (NAR) test is a color comparison method characterized by its simplicity, speed, and lack of dependence on electrical power for analysis. The objective of this study was to compare the sensitivity and specificity of these four rapid antibiotic test methods with the novel NAR test. A total of 28 samples were prepared, including 8 milk samples without residues spiked with milk containing Beta-lactam residues, 10 samples without residues, and 10 samples with known Beta-lactam (benzyl penicillin) residues. The four analysis methods were executed following their respective technical guidelines and manuals. For the NAR test, 50 mg of its active ingredient was mixed with 3 ml of milk, and color changes were observed. Results revealed the sensitivity and specificity of the NAR test to be 66.7% and 100%, respectively. On the other hand, each of the other four tests exhibited both a sensitivity and specificity of 100%. According to the Landis-Koch scale, the Kappa coefficient indicated a moderate level of agreement (0.5882) between the NAR test method and the other methods. Additionally, the odds ratio demonstrated a positive correlation between the NAR test and the four methods. The NAR test is particularly well-suited for implementation at milk collection points or on-farm levels prior to the bulk transportation stage.

Key words: Antibiotic residues, rapid test kit, Beta-lactam, Beta-lactamase, NAR test.

Appendix E: Publication on the Novel Platform Test Method

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African Journal of Food Science

Full Length Research Paper

A novel platform test to detect Beta-lactam residues in raw milk

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Microorganisms causing mastitis have developed resistance to Beta-lactam antibiotics due to the production of Beta-lactamase, an enzyme detectable in raw milk. This study aimed to establish a novel platform test for identifying Beta-lactam antibiotic residues in raw milk using the Hardy Diagnostic Beta-lactamase Test (HDBT) reagent. The HDBT reagent was modified by dissolving penicillin, sodium chloride, trisodium citric acid, trisodium phosphate, and phenol red in distilled water. Pooled raw milk samples were obtained from 3 Friesian and 3 Ayrshire lactating cows afflicted with subclinical mastitis and treated with Beta-lactam antibiotics. Various mixing ratios of the reagent were explored across nine levels, and the potential influence of cow breeds on test outcomes was also investigated. Trained assessors evaluated color distinctions between Beta-lactam positive and negative raw milk samples in all experimental trials. Results indicated that the gradual incorporation of trisodium phosphate and phenol red into the reagent led to a notable differentiation ($P \leq 0.05$) between Beta-lactam positive and negative raw milk samples. The ratio of 5:5 was identified as the most effective and significantly differed ($P \leq 0.05$) from the other ratios. However, the test method didn't demonstrate a significant discrepancy ($P \leq 0.05$) between raw milk samples from Friesian and Ayrshire cows. This method has potential application in assessing raw milk along collection routes to determine whether to accept, set aside, or reject milk suspected to contain antibiotic residues. A fuchsia purple color indicates a Beta-lactam negative sample, while a peach or pink color indicates a positive sample.

Key words: Raw milk, antibiotic residues, Beta-lactam, Beta-lactamase enzyme, trisodium phosphate.

Appendix F: Data on Ayrshire and Friesian Breed Comparison Results

Sample code	Analyst	Result
ABC	1	1.9
BCA	1	2.1
CBA	1	2.6
BBA	1	2.1
BAB	1	1.9
ABB	1	2.2
ABC	2	0.5
BCA	2	2.1
CBA	2	3
BBA	2	1.3
BAB	2	1.4
ABB	2	0.7
ABC	3	0.5
BCA	3	0.9
CBA	3	0.4
BBA	3	0.7
BAB	3	0.5
ABB	3	0.3
ABC	4	0.9
BCA	4	2.8
CBA	4	0.5
BBA	4	0.9
BAB	4	0.9
ABB	4	0.9
ABC	5	0.5
BCA	5	1.2
CBA	5	1.3
BBA	5	1.1
BAB	5	2.9
ABB	5	0.2
ABC	6	1.5
BCA	6	1.3
CBA	6	1.6
BBA	6	1.1
BAB	6	1.2
ABB	6	1.9
ABC	7	0.6
BCA	7	0.5
CBA	7	0.7
BBA	7	0.3
BAB	7	1.1
ABB	7	0.1
ABC	8	0.6
BCA	8	0.9
CBA	8	1
BBA	8	0.5
BAB	8	0.2
ABB	8	0.7
ABC	9	0.4
BCA	9	0.6
CBA	9	0.5
BBA	9	0.4
BAB	9	0.4
ABB	9	1.9
ABC	10	1.8
BCA	10	1.1
CBA	10	1.3
BBA	10	1.1
BAB	10	1.2
ABB	10	0.9

Appendix F: Ranking Methods Results in Best Reagent Selection

RANKING METHOD			
CODE	**REPLICATE**	**Analysts**	**Result**
ABC	1	1	10
BCA	1	1	15
CBA	1	1	20
BBA	1	1	25
BAB	1	1	30
ABB	1	1	5
ABC	1	2	10
BCA	1	2	15
CBA	1	2	25
BBA	1	2	20
BAB	1	2	30
ABB	1	2	5
ABC	1	3	15
BCA	1	3	10
CBA	1	3	20
BBA	1	3	25
BAB	1	3	30
ABB	1	3	5
ABC	1	4	10
BCA	1	4	15
CBA	1	4	20
BBA	1	4	25
BAB	1	4	30
ABB	1	4	5
ABC	1	5	25
BCA	1	5	20
CBA	1	5	15
BBA	1	5	10
BAB	1	5	30
ABB	1	5	5
ABC	1	6	10
BCA	1	6	25
CBA	1	6	20
BBA	1	6	15
BAB	1	6	30
ABB	1	6	5
ABC	1	7	10
BCA	1	7	15
CBA	1	7	25
BBA	1	7	20
BAB	1	7	30
ABB	1	7	5
ABC	1	8	15
BCA	1	8	10
CBA	1	8	20
BBA	1	8	25
BAB	1	8	30
ABB	1	8	5
ABC	1	9	10
BCA	1	9	15
CBA	1	9	25
BBA	1	9	20
BAB	1	9	30
ABB	1	9	5
ABC	1	10	10
BCA	1	10	20
CBA	1	10	15
BBA	1	10	25
BAB	1	10	30
ABB	1	10	5

Appendix H: Results for Line Scale Methods on Reagent Selection

LINE SCALE METHOD			
CODE	**REPLICATE**	**Analysts**	**Results**
ABC	1	1	3.5
BCA	1	1	5.1
CBA	1	1	4.6
BBA	1	1	6.4
BAB	1	1	14.9
ABB	1	1	0.1
ABC	1	2	3.9
BCA	1	2	3.6
CBA	1	2	3.9
BBA	1	2	3.4
BAB	1	2	14.6
ABB	1	2	0.4
ABC	1	3	2
BCA	1	3	3.4
CBA	1	3	5.2
BBA	1	3	5.9
BAB	1	3	14.5
ABB	1	3	0.2
ABC	1	4	3.2
BCA	1	4	1.2
CBA	1	4	2.7
BBA	1	4	2
BAB	1	4	13.7
ABB	1	4	0.3
ABC	1	5	1.4
BCA	1	5	0.4
CBA	1	5	0.6
BBA	1	5	1.2
BAB	1	5	13.6
ABB	1	5	0.2
ABC	1	6	0.8
BCA	1	6	0.7
CBA	1	6	0.8
BBA	1	6	6.1
BAB	1	6	13
ABB	1	6	0.5
ABC	1	7	0.8
BCA	1	7	1.2
CBA	1	7	1.8
BBA	1	7	1.5
BAB	1	7	14.7
ABB	1	7	0.2
ABC	1	8	1.3
BCA	1	8	1.6
CBA	1	8	2.7
BBA	1	8	0.8
BAB	1	8	14.2
ABB	1	8	0.3
ABC	1	9	3.6
BCA	1	9	2.8
CBA	1	9	2.2
BBA	1	9	2.6
BAB	1	9	14.1
ABB	1	9	0.3
ABC	1	10	2.4
BCA	1	10	0.9
CBA	1	10	3.1
BBA	1	10	4.3
BAB	1	10	14.5
ABB	1	10	0.3

ABC	2	1	3.3	BBA	2	6	6.2
BCA	2	1	4.9	BAB	2	6	14
CBA	2	1	4.2	ABB	2	6	0.6
BBA	2	1	6.1	ABC	2	7	0.8
BAB	2	1	14.9	BCA	2	7	1
ABB	2	1	0.08	CBA	2	7	1.5
ABC	2	2	3.1	BBA	2	7	1.2
BCA	2	2	3.2	BAB	2	7	14.6
CBA	2	2	3.4	ABB	2	7	0.2
BBA	2	2	3	ABC	2	8	1.1
BAB	2	2	14.1	BCA	2	8	1.5
ABB	2	2	0.1	CBA	2	8	2.3
ABC	2	3	1.8	BBA	2	8	0.5
BCA	2	3	3	BAB	2	8	14
CBA	2	3	4.8	ABB	2	8	0.2
BBA	2	3	5.4	ABC	2	9	3.4
BAB	2	3	14.4	BCA	2	9	2.1
ABB	2	3	0.2	CBA	2	9	1.9
ABC	2	4	3.1	BBA	2	9	2.3
BCA	2	4	1	BAB	2	9	14
CBA	2	4	2.4	ABB	2	9	0.1
BBA	2	4	1.8	ABC	2	10	2.1
BAB	2	4	13.5	BCA	2	10	0.6
ABB	2	4	0.1	CBA	2	10	2.8
ABC	2	5	1.2	BBA	2	10	4
BCA	2	5	0.2	BAB	2	10	14.2
CBA	2	5	0.5	ABB	2	10	0.1
BBA	2	5	1.1	ABC	3	1	3.8
BAB	2	5	13.5	BCA	3	1	5.4
ABB	2	5	0.3	CBA	3	1	4.9
ABC	2	6	0.7	BBA	3	1	6.7
BCA	2	6	0.8	BAB	3	1	15
CBA	2	6	0.6	ABB	3	1	0.3

ABC	3	2	4.1	BBA	3	6	6.3
BCA	3	2	3.8	BAB	3	6	13.2
CBA	3	2	4.1	ABB	3	6	0.7
BBA	3	2	3.6	ABC	3	7	1
BAB	3	2	14.9	BCA	3	7	1.4
ABB	3	2	0.6	CBA	3	7	2
ABC	3	3	2.2	BBA	3	7	1.7
BCA	3	3	3.6	BAB	3	7	14.9
CBA	3	3	5.4	ABB	3	7	0.4
BBA	3	3	6.1	ABC	3	8	1.5
BAB	3	3	14.7	BCA	3	8	1.8
ABB	3	3	0.3	CBA	3	8	2.9
ABC	3	4	3.6	BBA	3	8	1.2
BCA	3	4	1.6	BAB	3	8	14.6
CBA	3	4	3	ABB	3	8	0.7
BBA	3	4	2.3	ABC	3	9	4
BAB	3	4	14	BCA	3	9	3.2
ABB	3	4	0.6	CBA	3	9	2.6
ABC	3	5	1.7	BBA	3	9	3
BCA	3	5	0.7	BAB	3	9	14.5
CBA	3	5	0.9	ABB	3	9	0.7
BBA	3	5	1.5	ABC	3	10	2.9
BAB	3	5	13.9	BCA	3	10	1.4
ABB	3	5	0.4	CBA	3	10	3.6
ABC	3	6	1	BBA	3	10	4.8
BCA	3	6	0.9	BAB	3	10	15
CBA	3	6	1	ABB	3	10	0.8

Appendix G: Ranking and Line Scale Method Results for Breed Comparison

RANKING METHOD				LINE SCALE METHOD			
Reagent	Replicate	Analysts	Result	Reagent	Replicate	Analysts	Result
ABC	1	1	5	ABC	1	1	0.4
ABC	1	2	10	ABC	1	2	0.4
ABC	1	3	5	ABC	1	3	0.4
ABC	1	4	10	ABC	1	4	0.4
ABC	1	5	15	ABC	1	5	0.4
ABC	1	6	20	ABC	1	6	0.4
ABC	1	7	15	ABC	1	7	0.4
ABC	1	8	20	ABC	1	8	0.4
ABC	1	9	5	ABC	1	9	0.4
ABC	1	10	15	ABC	1	10	0.4
ABC	1	11	20	ABC	1	11	0.4
ABC	1	12	5	ABC	1	12	0.4
ABC	2	1	5	ABC	2	1	0.2
ABC	2	2	10	ABC	2	2	0.2
ABC	2	3	5	ABC	2	3	0.2
ABC	2	4	10	ABC	2	4	0.2
ABC	2	5	15	ABC	2	5	0.2
ABC	2	6	20	ABC	2	6	0.2
ABC	2	7	15	ABC	2	7	0.2
ABC	2	8	20	ABC	2	8	0.2
ABC	2	9	5	ABC	2	9	0.2
ABC	2	10	15	ABC	2	10	0.2
ABC	2	11	20	ABC	2	11	0.2
ABC	2	12	5	ABC	2	12	0.2
ABC	3	1	5	ABC	3	1	0.2
ABC	3	2	10	ABC	3	2	0.2
ABC	3	3	5	ABC	3	3	0.2
ABC	3	4	10	ABC	3	4	0.2

ABC	3	5	15	ABC	3	5	0.2
ABC	3	6	20	ABC	3	6	0.2
ABC	3	7	15	ABC	3	7	0.2
ABC	3	8	20	ABC	3	8	0.2
ABC	3	9	5	ABC	3	9	0.2
ABC	3	10	15	ABC	3	10	0.2
ABC	3	11	20	ABC	3	11	0.2
ABC	3	12	5	ABC	3	12	0.2
BCA	1	1	20	BCA	1	1	0.3
BCA	1	2	5	BCA	1	2	0.3
BCA	1	3	10	BCA	1	3	0.3
BCA	1	4	5	BCA	1	4	0.3
BCA	1	5	20	BCA	1	5	0.3
BCA	1	6	15	BCA	1	6	0.3
BCA	1	7	10	BCA	1	7	0.3
BCA	1	8	10	BCA	1	8	0.3
BCA	1	9	10	BCA	1	9	0.3
BCA	1	10	10	BCA	1	10	0.3
BCA	1	11	15	BCA	1	11	0.3
BCA	1	12	20	BCA	1	12	0.3
BCA	2	1	20	BCA	2	1	0.3
BCA	2	2	5	BCA	2	2	0.3
BCA	2	3	10	BCA	2	3	0.3
BCA	2	4	5	BCA	2	4	0.3
BCA	2	5	20	BCA	2	5	0.3
BCA	2	6	15	BCA	2	6	0.3
BCA	2	7	10	BCA	2	7	0.3
BCA	2	8	10	BCA	2	8	0.3
BCA	2	9	10	BCA	2	9	0.3
BCA	2	10	10	BCA	2	10	0.3
BCA	2	11	15	BCA	2	11	0.3
BCA	2	12	20	BCA	2	12	0.3

BCA	3	1	20	BCA	3	1	0.3
BCA	3	2	5	BCA	3	2	0.3
BCA	3	3	10	BCA	3	3	0.3
BCA	3	4	5	BCA	3	4	0.3
BCA	3	5	20	BCA	3	5	0.3
BCA	3	6	15	BCA	3	6	0.3
BCA	3	7	10	BCA	3	7	0.3
BCA	3	8	10	BCA	3	8	0.3
BCA	3	9	10	BCA	3	9	0.3
BCA	3	10	10	BCA	3	10	0.3
BCA	3	11	15	BCA	3	11	0.3
BCA	3	12	20	BCA	3	12	0.3
CBA	1	1	15	CBA	1	1	0.1
CBA	1	2	15	CBA	1	2	0.2
CBA	1	3	20	CBA	1	3	0.2
CBA	1	4	15	CBA	1	4	0.2
CBA	1	5	5	CBA	1	5	0.2
CBA	1	6	10	CBA	1	6	0.2
CBA	1	7	5	CBA	1	7	0.2
CBA	1	8	15	CBA	1	8	0.2
CBA	1	9	15	CBA	1	9	0.2
CBA	1	10	5	CBA	1	10	0.2
CBA	1	11	10	CBA	1	11	0.2
CBA	1	12	15	CBA	1	12	0.2
CBA	2	1	15	CBA	2	1	0.1
CBA	2	2	15	CBA	2	2	0.2
CBA	2	3	20	CBA	2	3	0.2
CBA	2	4	15	CBA	2	4	0.2
CBA	2	5	5	CBA	2	5	0.2
CBA	2	6	10	CBA	2	6	0.2
CBA	2	7	5	CBA	2	7	0.2
CBA	2	8	15	CBA	2	8	0.2

CBA	2	9	15	CBA	2	9	0.2
CBA	2	10	5	CBA	2	10	0.2
CBA	2	11	10	CBA	2	11	0.2
CBA	2	12	15	CBA	2	12	0.2
CBA	3	1	15	CBA	3	1	0.1
CBA	3	2	15	CBA	3	2	0.2
CBA	3	3	20	CBA	3	3	0.2
CBA	3	4	15	CBA	3	4	0.2
CBA	3	5	5	CBA	3	5	0.2
CBA	3	6	10	CBA	3	6	0.2
CBA	3	7	5	CBA	3	7	0.2
CBA	3	8	15	CBA	3	8	0.2
CBA	3	9	15	CBA	3	9	0.2
CBA	3	10	5	CBA	3	10	0.2
CBA	3	11	10	CBA	3	11	0.2
CBA	3	12	15	CBA	3	12	0.2
BAC	1	1	10	BAC	1	1	0.4
BAC	1	2	20	BAC	1	2	0.4
BAC	1	3	15	BAC	1	3	0.4
BAC	1	4	20	BAC	1	4	0.4
BAC	1	5	10	BAC	1	5	0.4
BAC	1	6	5	BAC	1	6	0.4
BAC	1	7	20	BAC	1	7	0.4
BAC	1	8	5	BAC	1	8	0.4
BAC	1	9	20	BAC	1	9	0.4
BAC	1	10	20	BAC	1	10	0.4
BAC	1	11	5	BAC	1	11	0.4
BAC	1	12	10	BAC	1	12	0.4
BAC	2	1	10	BAC	2	1	0.4
BAC	2	2	20	BAC	2	2	0.4
BAC	2	3	15	BAC	2	3	0.4
BAC	2	4	20	BAC	2	4	0.4

BAC	2	5	10	BAC	2	5	0.4
BAC	2	6	5	BAC	2	6	0.4
BAC	2	7	20	BAC	2	7	0.4
BAC	2	8	5	BAC	2	8	0.4
BAC	2	9	20	BAC	2	9	0.4
BAC	2	10	20	BAC	2	10	0.4
BAC	2	11	5	BAC	2	11	0.4
BAC	2	12	10	BAC	2	12	0.4
BAC	3	1	10	BAC	3	1	0.4
BAC	3	2	20	BAC	3	2	0.4
BAC	3	3	15	BAC	3	3	0.4
BAC	3	4	20	BAC	3	4	0.4
BAC	3	5	10	BAC	3	5	0.4
BAC	3	6	5	BAC	3	6	0.4
BAC	3	7	20	BAC	3	7	0.4
BAC	3	8	5	BAC	3	8	0.4
BAC	3	9	20	BAC	3	9	0.4
BAC	3	10	20	BAC	3	10	0.4
BAC	3	11	5	BAC	3	11	0.4
BAC	3	12	10	BAC	3	12	0.4
ABB	1	1	25	ABB	1	1	12.5
ABB	1	2	25	ABB	1	2	13
ABB	1	3	25	ABB	1	3	14
ABB	1	4	25	ABB	1	4	12.9
ABB	1	5	25	ABB	1	5	12.5
ABB	1	6	25	ABB	1	6	13.7
ABB	1	7	25	ABB	1	7	14.6
ABB	1	8	25	ABB	1	8	13.9
ABB	1	9	25	ABB	1	9	12.5
ABB	1	10	25	ABB	1	10	14.9
ABB	1	11	25	ABB	1	11	14.5
ABB	1	12	25	ABB	1	12	14.8

ABB	2	1	25	ABB	2	1	14.7
ABB	2	2	25	ABB	2	2	13.7
ABB	2	3	25	ABB	2	3	12
ABB	2	4	25	ABB	2	4	13.6
ABB	2	5	25	ABB	2	5	14.7
ABB	2	6	25	ABB	2	6	13.8
ABB	2	7	25	ABB	2	7	14.5
ABB	2	8	25	ABB	2	8	13.9
ABB	2	9	25	ABB	2	9	14.7
ABB	2	10	25	ABB	2	10	14.4
ABB	2	11	25	ABB	2	11	14.7
ABB	2	12	25	ABB	2	12	14.7
ABB	3	1	25	ABB	3	1	13.9
ABB	3	2	25	ABB	3	2	13
ABB	3	3	25	ABB	3	3	13.9
ABB	3	4	25	ABB	3	4	13.3
ABB	3	5	25	ABB	3	5	13.9
ABB	3	6	25	ABB	3	6	13.9
ABB	3	7	25	ABB	3	7	14.8
ABB	3	8	25	ABB	3	8	13.7
ABB	3	9	25	ABB	3	9	13.8
ABB	3	10	25	ABB	3	10	14.8
ABB	3	11	25	ABB	3	11	14.9
ABB	3	12	25	ABB	3	12	14.9

Appendix H: Line Scale Results for the Powder and the Reagent Comparison

LINESCALE				1	3	5	0.2
METHOD				1	3	6	0.2
Method	Replicate	Analysts	Result	1	3	7	0.2
1	1	1	0.4	1	3	8	0.2
1	1	2	0.4	1	3	9	0.2
1	1	3	0.4	1	3	10	0.2
1	1	4	0.4	1	3	11	0.2
1	1	5	0.4	1	3	12	0.2
1	1	6	0.4	2	1	1	0.3
1	1	7	0.4	2	1	2	0.3
1	1	8	0.4	2	1	3	0.3
1	1	9	0.4	2	1	4	0.3
1	1	10	0.4	2	1	5	0.3
1	1	11	0.4	2	1	6	0.3
1	1	12	0.4	2	1	7	0.3
1	2	1	0.2	2	1	8	0.3
1	2	2	0.2	2	1	9	0.3
1	2	3	0.2	2	1	10	0.3
1	2	4	0.2	2	1	11	0.3
1	2	5	0.2	2	1	12	0.3
1	2	6	0.2	2	2	1	0.3
1	2	7	0.2	2	2	2	0.3
1	2	8	0.2	2	2	3	0.3
1	2	9	0.2	2	2	4	0.3
1	2	10	0.2	2	2	5	0.3
1	2	11	0.2	2	2	6	0.3
1	2	12	0.2	2	2	7	0.3
1	3	1	0.2	2	2	8	0.3
1	3	2	0.2	2	2	9	0.3
1	3	3	0.2	2	2	10	0.3
1	3	4	0.2	2	2	11	0.3

2	2	12	0.3	2	3	7	0.3
2	3	1	0.3	2	3	8	0.3
2	3	2	0.3	2	3	9	0.3
2	3	3	0.3	2	3	10	0.3
2	3	4	0.3	2	3	11	0.3
2	3	5	0.3	2	3	12	0.3
2	3	6	0.3				

Appendix I: ANOVA Tables

Table 1: The ANOVA table showing the main factors and the effect of different dependent variables of the results

SOV	D.o.F	Results
Code	5	760.19^{***}
Person	9	13.81^{***}
Error	165	1.05^{***}
CV	-	24.44
R^2	-	95.76

Key: SOV= Source of variation; DoF = Degree of Freedom; CV = Coefficient of Variation; and R^2 = Coefficient of Determination.

Table 2: The ANOVA table showing the main factors and the effect of different dependent variables of the results

SOV	D.o.F	Results
Method	1	0.001^{ns}
Person	11	0.001^{ns}
Error	59	0.001^{ns}
CV	-	24.24
R^2	-	17.21

Key: SOV= Source of variation; D.o.F = Degree of Freedom; CV = Coefficient of Variation; and R^2 = Coefficient of Determination.

Table 3: The ANOVA table showing the main factors and the effect of different dependent variables of the results

SOV	D.o.F	Results
Reagent	4	1342.67^{***}
Person	11	0.22^{ns}
Error	164	0.13^{***}
CV	-	11.75
R^2	-	99.62

Key: SOV= Source of variation; DoF = Degree of Freedom; CV = Coefficient of Variation; and R^2 = Coefficient of Determination.

Table 4: The ANOVA table showing the main factors and the effect of different dependent variables of the results

SOV	**D.o.F**	**Results**
Code	5	760.19^{***}
Person	9	13.81^{***}
Error	165	1.05^{***}
CV	-	24.44
R^2	-	95.76

Key: SOV= Source of variation; DoF = Degree of Freedom; CV = Coefficient of Variation; and R^2 = Coefficient of Determination.

Table 5: The ANOVA table showing the main factors and the effect of different dependent variables of the results

SOV	D.o.F	Results
Code	5	0.34^{ns}
Person	9	1.56^{***}
Error	45	0.32^{***}
CV	-	51.35
R^2	-	51.84

Key: SOV= Source of variation; DoF = Degree of Freedom; CV = Coefficient of Variation; and R^2 = Coefficient of Determination.

Table 6: The ANOVA table showing the main factors and the effect of different dependent variables of the results

SOV	**D.o.F**	**Results**
Code	2	155.09^{***}
Replicate	2	0.11^{ns}
Person	11	7.00^{***}
Error	92	1.85^{***}
CV	-	30.02
R^2	-	94.92

Key: SOV= Source of variation; DoF = Degree of Freedom; CV = Coefficient of Variation; and R^2 = Coefficient of Determination.

Appendix K: National Commission for Science, Technology and Innovation (NACOSTI)

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 737493

Date of Issue: 20/July/2020

RESEARCH LICENSE

This is to Certify that Ms.. TERESIAH Wangui NDUNGU of Egerton University, has been licensed to conduct research in Nakuru on the topic: DEVELOPMENT OF A PLATFORM TEST TO DETECT ?-LACTAM RESIDUES IN RAW MILK for the period ending : 20/July/2021.

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Director General

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

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Appendix L: Innovation Registration Certificate at Kenya Industrial Property Institute

Kenya Industrial Property Institute

The Industrial Property Act, 2001

CERTIFICATE

OF REGISTRATION OF UTILITY MODEL

It is hereby certified that a utility model with utility model number **375** has been registered in the name **NDUNGU TERESIAH WANGUI of P. O. BOX 63-20114 KABAZI, Kenya** in respect of an invention disclosed in an application number **KE/U/2020/1473** having a date of filing of **29/10/2020** and being an invention titled **METHOD AND PRODUCT FOR ANTIBIOTIC RESIDUES DETECTION IN RAW MILK.**

Dated at **Nairobi** this **17th day of December, 2021.**

.....

John Onyango
Ag. Managing Director

Appendix M: Cost Analysis

Costs of ingredients							**Requirement for the powder**		
	Quantity	Unit cost	Total cost				**S/no.**	Ingredient	Amount (g)/L of H_2O
REAGENTS							1	Penicillin	15
Penicillin (8pcs for one test)	10	150	1500				2	Sodium Chloride	5
Trisodium Citric acid (500g)	1	2000	2000				3	Trisodium Citric Acid	1.5
Trisodium phosphate (500g)	1	2000	2000				4	Trisodium Phosphate	10.48
Phenol red indicator (25g)	1	1800	1800				5	Phenol Red	0.08
Sodium chloride (500g)	1	800	800						32.06
			8100						
Quantity of powder per single test									
1 test requires 0.05g in 3ml of milk									
This can further reduce after improved packaging									
				Quantity (g)	Unit cost	Total			

						cost			
Penicillin (pcs)	10	50	500	8	50	400.0			
Trisodium Citric acid (500g)	1	2000	2000	1.5	6	9.0			
Trisodium phosphate (500g)	1	2000	2000	10.48	41.92	439.3			
Phenol red indicator (25g)	1	1800	1800	0.08	5.76	0.5			
Sodium chloride (500g)	1	800	800	5	8	40.0			
						888.8			
Total grams used in making the powder per time			32.06						
Total number of tests that can be carried out			641						
Total cost per test			1.39	Minus labour, packaging cost plus sale of innovation					
Labor	1 bob per test?		1						
Packaging	20bob per test?		20						
Innovation fee	2 bob per test?		2						

Total cost per test after additions			24.39						
			25	Approximate cost per test					