



**UNIVERSIDADE  
E D U A R D O  
MONDLANE**

**Faculty of Veterinary Science**

**Master in Food Safety**

**ANTIBIOTIC RESIDUES IN RAW COW MILK COLLECTED FROM SMALLHOLDER  
DAIRY PRODUCERS IN KASAMA AND MBALA, ZAMBIA**

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**MAPUTO**

**February 16, 2026**



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## **DECLARATION OF ORIGINALITY OF THE PROJECT**

I declare that this dissertation titled “Antibiotic residues in raw cow milk collected from smallholder dairy producers in Kasama and Mbala, Zambia,” has never been submitted for the purpose of obtaining any degree or in any other field and that it is the result of my individual labour.

This dissertation is presented in partial fulfilment of the requirements for obtaining the degree of Masters in Food Safety, from the University of Eduardo Mondlane.

Date: ...../...../.....

Signature

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(Goliath Eneya Zulu)

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## **DEDICATION**

I dedicate this dissertation to my beloved parents, whose unwavering support and guidance have shaped my journey. To my wonderful and beautiful wife (Loveness Nankamba Zulu) and two sons (Joshua Kachinga and Johnathan Takondwa Zulu), thank you for your love, encouragement, and patience throughout this endeavour. I also extend my heartfelt dedication to my sister (Doris Kondwani Zulu) and my overall family and friends, whose encouragement has been invaluable during this journey. Lastly, I dedicate this work to almighty God, whose grace, blessings and love have made all things possible.

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## LIST OF ABBREVIATION AND ACRONYMS

$\mu$ l	: Microliter
ACF	: Agriculture Consultative Forum
AMR	: Antimicrobial Resistance
AMU	: Antimicrobial use
AR	: Antimicrobial residues
ASF	: Animal-source food
CAADP	: Comprehensive Africa Agriculture Development Programme
CAC	: Codex Alimentarius Commission
CBPP	: Contagious Bovine Pleuropneumonia
CI	: Confidence interval
cpm	: Count Per Minutes
CVRI	: Central Veterinary Research Institute
EC	: European commission
EFSA	: European Food Safety Authority
<i>et al</i>	: And others
EU	: European Union
FAO	: Food Agriculture Organisation
FMD	: Foot and Mouth Disease
GAIN	: Global Alliance for Improved Nutrition
GHDx	: Global Health Data Exchange
HLB	: Hydrophilic-Lipophilic Balance
HPLC	: High Performance Liquid Chromatography
i.e.	: That is
kBq	: Kilobecquerels
LC	: Liquid Chromatography
LFM	: Laboratory Fortified Matrix
LOD	: Limit of Detection
LOQ	: Limit of Quantification
LRB	: Laboratory Reagent Blank
MCC	: Milk collection centres
mL	: Millilitre
MRLs	: Maximum Residue Limits
MS	: Mass spectrometer

NEPAD	: New Partnership for Africa's Development
NGOs	: Non-governmental Organisations
ODK	: Open Data Kit
ppb	: Parts per billion
rpm	: Revolution per minute
rpm	: Revolution per minute
SDGs	: Sustainable Development Goals
SPE	: Solid Phase Extraction cartridge
TLC	: Thin Layer Chromatography
UV	: Ultra-Violet
WHO	: World Health Organisation
ZABS	: Zambia Bureau of Standards

## ABSTRACT

**Background:** Antimicrobials have been used in livestock production for several purposes, including growth promotion, preventive, metaphylactic, and therapeutic. The misuse and overuse of antibiotics in animal-source foods is widely recognized as a key driver of AMR (public health threat) in humans. **Objective:** Assess antibiotic class residues in raw cow milk from smallholder producers in Mbala and Kasama, Zambia. **Materials and methods:** Cross-sectional study among randomly collected samples (54 Mbala and 39 Kasama) between May-June, 2025 were screened for beta-lactams, tetracyclines, macrolides, sulfonamides and aminoglycosides by Charm II assay. Data analysis using Stata/SE 14.2, at 95% CI ( $p=0.05$ ) employed; Pearson Chi-square, Pearson correlation, and multivariable linear regression. Semi-structured questionnaires were administered to 101 respondents, 41.6% and 58.4% from Mbala and Kasama respectively on KoboToolbox, to assess knowledge, attitude and practices (KAP) regarding antibiotic use and residues. **Results:** Overall, 91.4% of samples contained antibiotic class residues. 82.1% and 98.1% from Kasama and Mbala, respectively ( $p=0.006$ ), had residues above EU/MRLs (screened positive). Sulfonamides had 68.8%, macrolides 58.1%, tetracyclines 12.9%, beta-lactams 9.7% and aminoglycosides 2.2%. 52.5% of respondents in Kasama had good knowledge, 57.6% positive attitude and 40% good practices. In Mbala 61.9% had good knowledge, 47.6% positive attitude and 35.7% good practices. Knowledge scores ( $p=0.350$ ), attitude ( $p=0.320$ ), and practices ( $p=0.738$ ) were not significant between districts. Only Mbala had correlation between knowledge-attitude ( $r=0.687$ ,  $p<0.001$ ), knowledge-practice ( $r=0.470$ ,  $p=0.002$ ) and attitude-practice ( $r=0.629$ ,  $p<0.001$ ). **Conclusion:** Revealed that farmer's lack of proper KAP leads to antibiotic misuse, posing public health concerns and requires urgent strategies to be addressed.

**Key words:** Antimicrobials; Animal-source foods; Antimicrobial resistance; Charm II receptor assay; Dairy; One health.

## RESUMO

**Enquadramento:** Os antimicrobianos têm sido utilizados na produção pecuária para diversos fins, incluindo a promoção do crescimento, prevenção, metafilaxia e tratamento. O uso indevido e excessivo de antibióticos em alimentos de origem animal é amplamente reconhecido como um fator chave da resistência antimicrobiana (RAM) em humanos. **Objectivo:** Avaliar os resíduos de diferentes classes de antibióticos no leite cru de vacas de pequenos produtores de Mbala e Kasama, Zâmbia. **Materiais e métodos:** Estudo transversal com amostras recolhidas aleatoriamente (54 em Mbala e 39 em Kasama) entre maio e junho de 2025, analisadas para deteção de beta-lactâmicos, tetraciclina, macrolídeos, sulfonamidas e aminoglicosídeos pelo ensaio Charm II. A análise dos dados foi realizada com recurso ao software Stata/SE 14.2, com um intervalo de confiança de 95% ( $p=0,05$ ); foram empregues os testes Qui-quadrado de Pearson, correlação de Pearson e regressão linear multivariada. Foram aplicados questionários semiestruturados a 101 respondentes, sendo 41,6% de Mbala e 58,4% de Kasama, através da plataforma KoboToolbox, para avaliar o conhecimento, as atitudes e as práticas (CAP) em relação ao uso e aos resíduos de antibióticos. **Resultados:** No geral, 91,4% das amostras continham resíduos de classes de antibióticos. Em Kasama, 82,1% e Mbala, respetivamente ( $p=0.006$ ), apresentaram resíduos acima dos LMRs (Limites Máximos de Resíduos) da UE (triagem positiva). As sulfonamidas representaram 68,8% das amostras, os macrolídeos 58,1%, as tetraciclina 12,9%, os beta-lactâmicos 9,7% e os aminoglicosídeos 2,2%. Em Kasama, 52,5% dos inquiridos demonstraram bons conhecimentos, 57,6% atitude positiva e 40% boas práticas. Em Mbala, 61,9% apresentaram bons conhecimentos, 47,6% uma atitude positiva e 35,7% boas práticas. Não houve diferença significativa entre os distritos nos escores de conhecimento ( $p=0.350$ ), atitude ( $p=0.320$ ) e práticas ( $p=0.738$ ). Apenas Mbala apresentou correlação entre o conhecimento-atitude ( $r=0.687$ ,  $p<0.001$ ), o conhecimento-prática ( $r=0.470$ ,  $p=0.002$ ) e a atitude-prática ( $r=0.629$ ,  $p<0.001$ ). **Conclusão:** Os resultados revelaram que a falta de conhecimentos, atitudes e práticas (CAP) adequadas entre os agricultores leva ao uso indevido de antibióticos, representando um problema de saúde pública que exige estratégias urgentes.

**Palavras-chave:** Antimicrobianos; Alimentos de origem animal; Resistência antimicrobiana; Ensaio do receptor Charm II; Laticínios; Saúde única.

# 1. INTRODUCTION

## 1.1 Motivation

The dairy sector is among the largest and fastest-growing segment of agriculture globally with an engagement of about 150 million farm households focused in milk production, i.e. more than 750 million people, the majority of who are in developing countries (FAO, 2010). In 2023, global milk production reached 965.7 million tonnes. Meanwhile, milk production in Africa reached 53.8 million tonnes in the same year (FAO, 2023). According to Mumba (2012) in Agriculture Consultative Forum report (ACF), Zambia had around 3000-4000 dairy producers in 2012 ranging from traditional to commercial producers, contributing approximately 253 million litres of milk to the national production capacity.

In Zambia, the traditional dairy value chain accounts for a large share of total milk supply than the commercial sector (Phiri *et al.*, 2021). The dairy production is an essential component of economic and sustainable development in terms of food and nutritional security, employment and income generation (Mainda *et al.*, 2015). It is estimated that smallholder dairy producers in Zambia contribute about 50% of the marketed milk (150 million litres), while 23% is supplied by large scale commercial farmers, with the remaining 27% being imported as milk and milk products like powdered milk, yogurt and ice-cream (NEPAD, 2004).

Antibiotics are natural or synthetic chemical substances that are produced to kill or inhibit microorganism growth. Antimicrobials have been utilised in livestock farming for several uses namely; therapeutic, metaphylactic or prophylactic and as growth promoters (World Health Organization, 2001). Over the recent past antibiotics have been widely used in the food production industry, and their utilization has increased tremendously due to misuse and lack of awareness among the users (Hossain *et al.*, 2023). Around 75% of the 12 million kilograms of antibiotics consumed globally each year are used to address infectious diseases, while the rest are utilised for preventive measures or growth promotion (Van Boeckel *et al.*, 2015).

In the daily application of antibiotics in both human and veterinary medicine there has been a growing global attention towards the development of antimicrobial resistance (AMR) due to misuse (Livermore, 2003). Misuse of antibiotics in livestock production leads to the presence of antibiotic residues in animal source foods (ASF) like milk, meat, poultry, fish and eggs which is associated with development of AMR (Barros *et al.*, 2023). A high prevalence of disease in cattle especially of tick-

borne disease such as Theileriosis, Anaplasmosis and Heart water in Sub-Saharan Africa including Zambia has made therapeutic use of antimicrobials, especially oxytetracycline and sulfadimidine has become of great concern because of common use (Mainda *et al.*, 2015).

Globally, as well as in Africa, there have been numerous reports of antibiotic residues in ASF due to the indiscriminate use of antibiotics in terms of adherence to antibiotic withdraw period. In many African countries, these residues often surpass the maximum residue limits (MRLs) set by the WHO (Darwish *et al.*, 2013). A study conducted by Mudenda *et al.* (2022) described that inappropriate dispensing and use of antibiotics in animals has contributed to deposition of antibiotic residues in ASF. The presence of antibiotics residues in ASF above the MRLs has been recognized globally by various authorities due to public health threats (Kempe *et al.*, 2000).

The presence of antibiotic residues in ASF is likely to cause; toxicity of bone marrow, destruction of microflora in human intestinal microflora, allergic reactions and cancer. In addition, may result into development and sustenance of antibiotic-resistant bacteria and genes (Cinquina *et al.*, 2003). AMR represents a global challenge (WHO, 2021). Globally 4.95 million people who died in 2019 suffered from drug-resistant infections. AMR directly caused 1.27 million of those deaths (WHO, 2019). According to WHO (2019), Zambia reported 3,700 deaths attributable to AMR and 15,600 deaths associated with AMR.

The economic burden per year due to healthcare costs and productivity losses is estimated in the European Union (EU) at EUR 1.5 billion and \$55 billion in the United states (Mobarki *et al.*, 2019; WHO, 2019). AMR is among the top 10 global public health threats (WHO, 2019). A report from EU in January, 2022 and the Member States identified AMR as one of the top three priority health threats (Simjee & Ippolito, 2022). Antibiotic residues in milk are slowly deteriorating the dairy industry by altering the results of analyses, inactivate the activity of starter cultures in cheese, yogurts production and compromising the production of acids (Berruga *et al.*, 2016; Gajda *et al.*, 2018).

Considering an alert to the crisis of AMR and its global public health threat, in 2015 the World Health Assembly adopted a Global Action Plan (GAP) on AMR through implementation of five main objectives (World Health Assembly, 2015). Among these objectives GAP targets to strengthen the knowledge and evidence base through surveillance and research (World Health Assembly, 2015). GAP also targets to improve awareness and understanding of AMR through effective communication,

education and training. In 2017, Zambia adopted from GAP and developed a multi-sectoral national action plan using a "One Health" approach to address AMR (Kapona, 2017).

Zambia has limited information on antibiotic usage and residues, poor regulation and surveillance protocols of antibiotics and poor regulatory controls (Mainda *et al.*, 2015). The findings from this study provide essential evidence for policy making to tackle the global issue of AMR associated with antibiotic residues in milk. This basic research was conducted as part of core strategies to address the challenge of antimicrobial residues (ARs) in ASF through surveillance. This study aimed to assess antibiotic residues in collected milk samples among smallholder dairy producers in Mbala and Kasama, Zambia with a view to ascertain its food safety and compliance with established MRLs.

## **1.2 Research problem**

In Africa mainly developing countries there has been high disease burden, indiscriminate antibiotic use and limited; surveillance protocol for antibiotic residues, awareness, inadequate regulations and knowledge, attitude and practices (KAP) leading to deposition of antibiotics in ASF such as milk, eggs, poultry meat and fish, leading to AMR (Chowdhury *et al.*, 2015; Mohamed *et al.*, 2020). Indiscriminate use of antibiotics constitutes a health hazard for the Zambian population (Nchima *et al.*, 2017). Majority of developing countries in Africa have inadequate detection facilities and the absence of an effective monitoring system for food residues, are significant factors contributing to the increased risk of antibiotic residues in milk (Kebede *et al.*, 2014).

In many low income countries, the use of antimicrobials for treatment of disease still remains undocumented and unregulated (Gilbert *et al.*, 2020). In Zambia, there has been limited data on antibiotic use and, residues and surveillance protocols on antibiotic residues in ASF (Mainda *et al.*, 2015). The nature and consequences of antimicrobial use (AMU) in animal production systems in Zambia are still ill-defined (Mainda, 2016). Moreover, only one study reported in Zambia which evaluated antibiotic residues in bulk raw cow milk from smallholder dairy producers (Kunda, 2015). There is no information on antibiotic residues in milk or any other food derived from animals in Zambia's northern region, specifically in Mbala and Kasama district. This situation indicates a significant lack of surveillance data on antibiotic residues in milk.

The AMR microorganisms evolving in dairy production have a significant potential to spread to humans through contamination of ASF and direct contact. However, the magnitude of this problem

in Zambia is unknown (Mainda, 2016). This basic research in nature aimed at assessing antibiotic residues in raw cow milk collected from smallholder dairy producers following a research gap as described by (Mainda *et al.*, 2015).

### **1.3 Objectives**

#### **1.3.1 General Objective**

To assess the presence of antibiotic residues in raw cow (bovine) milk collected from smallholder dairy producers in some parts of Kasama and Mbala districts, Zambia.

#### **1.3.2 Specific objectives**

The specific objectives of the study are to:

- To assess the presence of antibiotic classes residues in the milk samples.
- To assess the level of knowledge, attitude and practices regarding antibiotic use and residues among smallholder dairy producers.

#### **1.3.3 Research questions**

- What are the classes of antibiotic residues present in the milk samples?
- What is the level of knowledge, attitude and practices regarding antibiotic use and residues among smallholder dairy producers?

#### **1.3.4 Contribution of the research**

The outcomes of this research provided scientific based evidence and insights necessary for developing and implementing policies, regulations and strategies aimed at minimizing ARs in ASF especially milk. This study was conducted with a purpose to support and assure the enforcement of relevant legislation that promote responsible antibiotic use and ensure food safety among consumers. Findings of this study provided a basis for raising awareness among smallholder dairy producers and other livestock producers to promote good knowledge, positive attitude and good practices regarding antibiotic use and residues.

Awareness can be raised through training, communication, and educational programs, fostering a behavioural shift that promotes prudent antibiotic use and a better understanding about the public health consequences of antibiotics residues in ASF (milk). A well informed and knowledgeable livestock farmer is expected to have a behavioural change that promotes prudent use of antibiotics

during production thereby, reducing antibiotic residue deposition in ASF (milk). A study conducted by Nchima *et al.* (2017) described that rational use of antibiotics and control of antibiotic residue deposition in ASF is essential to the reduction of AMR in both humans and animals.

## 2. LITERATURE REVIEW

### 2.1 Milk and its composition

Milk is an opaque fluid secreted by female mammals for nourishment of their young which an excellent source of minerals, vitamins, proteins and sugar, for proper human and animal growth. It is considered one of the most consumed nutritious foods in the market and along with dairy products remains essential for the human diet (Pereira, 2014). The consumption of milk and its products has been documented to exhibit some cholesterol lowering effect thereby preventing incidences of atherosclerosis and coronary heart disease (Schmidt & Vleck, 2005). Common consumed milk products amongst smallholder dairy producers is locally fermented milk (Mumba, 2012).

Milk is the first food for mammals and provides all the necessary energy and nutrients to ensure proper growth, development and crucial in bone mass formation. Foods of dairy origin are generally considered as balanced and nutritive foods, being frequently included as important components of a healthy diet (Pereira, 2014). Despite, cow milk being probably the most frequently consumed, milk produced by sheep and goat is also consumed. Currently, several milk products can be found everywhere and the term “milk” generally refer to cow’s milk, produced by healthy animals and excluding the lactic secretion, until it is almost completely free of colostrum composition (Godden, 2008).

The definition of milk should exclude completely all other named milk products from vegetable origins such as “soya milk” and “almond milk”. These should be called “beverages” based on their origin, such as “soya beverage” as previewed in the European council legislation report (European Council, 2011). The chemical composition of milk can be influenced by several factors such as the environmental conditions, animal species and genetics, lactation stage and animal nutritional status (Caroli *et al.*, 2009; Kalač & Samková, 2010). A comparative review by Raynal-Ljutovac *et al.* (2008) indicated that sheep milk had a higher protein and fat content while goat milk had presented with a higher amount of A, B1 and B12 vitamins.

In addition, goat milk was reported to have an upper calcium and phosphorus content when compared to cow and sheep milk. On average the composition of milk is about 87% water, 4-5% lactose, 3% protein, 3-4% fat, 0.8% minerals and 0.1% vitamins (Haug *et al.*, 2007; Lindmark-Månsson *et al.*, 2003). The most common breeds of indigenous cattle used in Zambia include Angoni, Barotse and Tonga (Hofer, 2015). However, there are some exotic breeds (Friesian, Holstein and Jersey) and

crosses between these breeds and local. Highly managed breeds like exotic and crosses demand sufficient management and feeding to have a desired milk yield (Olofsson, 2013).

Table 1: Average nutritional composition of whole, low fat and skim milk (UHT).

<b>Composition (100 g)</b>	<b>Whole</b>	<b>Low Fat</b>	<b>Skim</b>
Energy (kcal)	62	47	34
Water (g)	88.1	89.1	90.5
Protein (g)	3	3.4	3.3
Fat (g)	3.5	1.6	0.2
Carbohydrates (g)	4.7	4.9	4.9
Cholesterol (mg)	13	8	1
Vitamin A (g)	59	22	0
Vitamin D (g)	0.05	0.05	0
Vitamin B1 (mg)	0.04	0.04	0.05
Vitamin B2 (mg)	0.14	0.11	0.05
Na (mg)	43	41	41
Ca (mg)	109	112	114
Mg (mg)	9	9	10

Source of Table 1 which reports on the average nutritional composition of whole, low fat and skim milk (UHT): Pereira (2014)

## **2.2 Position of the smallholder dairy sector globally**

Around 750 million people are involved in production of milk globally, the majority of whom are in developing countries and these reside on 150 million farm households. Based on milk equivalents, the average per capita global milk consumption amounts to around 200kg of milk per year, bearing very significant differences between countries or regions. The per capita consumption of milk in Western Europe is more than 300 litres of milk per year compared to some African and Asian countries which are less than 30 litres to as little as 10 litres sometimes (FAO, 2010).

The dairy industry provides a source of income and employment to many especially poor people across the globe. According to Ngongoni *et al.* (2006) venturing into smallholder dairy farming helps

people to obtain cash flow and promote poverty alleviation. An increase of milk production at household level assures household food and nutrition security, empowers women and youths in income generation ventures and other agriculture development. Therefore, the dairy sector is being promoted as part of the agenda to attain food security. It is essential that production of milk and other ASF and their products are safe or free from antibiotic residues that are at or above MRLs.

### **2.3 Position of the smallholder dairy sector in Zambia**

In Zambia the cattle industry is by far the most important component of the livestock sector, from an economic and cultural perspective. Over the recent past the Government of the Republic of Zambia has promoted economic growth, job creation, income generation, food and nutritional security and to reduce the high levels of poverty in the country. This has been through the development of a more competitive, diversified and sustainable dairy sector. The government has also promoted the growth of the dairy sub-sector through establishment of dairy cooperatives, Milk Collection Centres (MCC) and processing plants and distribution of dairy animals to its members.

This establishment has the potential to contribute to these developmental goals the country envisions (Mumba *et al.*, 2013). Smallholder dairy production promotes and facilitates job creation, income generation and contributes to food and nutritional security (Mumba, 2012). The majority of the milk produced by traditional farmers is either consumed by the household or sold in informal rural markets and consumed as raw milk. Some traditional small-scale producers sell their milk to locally established MCC who in turn sell either to processors or directly to consumers (Mumba, 2012).

In Zambia smallholder dairy is practiced in a production system that integrates crop and dairy farming (Mumba, 2012). The dairy sub-sector in Zambia contributes a major part in the livestock sector. There is estimation that smallholder dairy producers contribute about 50% of the marketed milk, large scale commercial farmer's supply 23%, while the remaining 27% is imported as milk and milk products according to a report by (NEPAD, 2004). In addition, livestock production is one of the major agriculture activities in Zambia with a total contribution of about 23% per capita supply of protein and 61% of this is from cattle through beef and milk supply (Robinson *et al.*, 2007).

According to a report by ACF of 2012, Mumba (2012) stated that Zambia has approximately 3,000-4,000 dedicated smallholder and commercial dairy farmers who produce milk for the formal market through the use of pure and cross breed dairy cattle. A report by GAIN (2018) stated that milk consumption in Zambia is 19-30kg/year per person, which is low for Sub-Saharan Africa (average

30.2 kg/year and far below the WHO recommendation of 175 kg/year. The dairy processors are facing a shortfall in the supply of raw milk due to a growing domestic demand for dairy products and low production amongst dairy producers.

According to a report by ACF of 2012, dairy processing industries are not utilising their current processing capacity translating to a capacity utilisation of about 59% and below (Mumba, 2012). The production of low milk yield among smallholder dairy producers may be that adoption rate of good animal husbandry practices (dipping, deworming, supplementary feeding) is still low and veterinary input supply and extension service are not consistent. There has been a challenge of animal diseases such as East coast fever (ECF), Food and Mouth Disease (FMD) and Contagious Bovine Pleuropneumonia (CBPP), (Mumba *et al.*, 2013).

Furthermore, Mumba (2012) suggest that the smallholder dairy enterprise in Zambia is somewhat a viable venture and could contribute to a reduction in rural poverty and unemployment, creation of wealth and enhancing household nutrition and food security. However, this venture is yet faced with several other challenges other than the ones mentioned above such as; long distances to the MCC, markets, lack of resources and a scarcity of high milk yielding dairy breeds and bullying services to increase dairy herd sizes.

According to Mumba (2012) ACF report estimated that out of a total national production of 253 million litres of milk produced in Zambia, there is only about 44 million litres that passes through formal market channels. The traditional sector produces a substantial portion of raw milk in Zambia which somewhat does not reach the formal market.

#### **2.4 Classification of antibiotics (anti-bacterial agents)**

Bacterial pathogens causing infectious disease in humans and animals represent a greater percentage of public health concern. In addition, antibiotics are part of 'antimicrobials' which include anti-fungal, anti-parasitic and anti-viral drugs used. Generally antimicrobials can be classified based on type of action: bacteriostatic and bactericidal (Ullah & Ali, 2017). According to Ullah & Ali (2017) antibiotics can be classified into five (5) main groups based on; type of action, source, spectrum of activity, chemical structure and function.

Classification of these antibiotics is based on different ways; however, the commonest classification is based on their molecular structure, mechanism of action and spectrum of activity (Calderón &

Sabundayo, 2007). Antibiotics can either be natural or synthetic where natural antibiotics are derived from natural sources, primarily fungi and bacteria (penicillin, tetracycline, erythromycin, streptomycin) and synthetic are chemically manufactured in laboratories (sulphonamide's, quinolones, cephalosporin's).

Antibiotics can also be classified according to; route of administration and killing effect or inhibits the growth of a microorganism (Fomnya *et al.*, 2021). Penicillin is a commonly used drug for humans and several animals and is considered one of the first discovered and used drugs. Similar to penicillin's, cephalosporin's, tetracyclines, aminoglycosides and sulphonamides and trimethoprim are common drugs used (Croubels & Daeseleire, 2012).

Table 2: Common use and mechanism(s) of action of antibiotic groups.

<b>Antibiotic Group</b>	<b>Examples</b>	<b>Common use (against)</b>	<b>Mechanism(s)</b>
Aminoglycosides	Gentamicin, Kanamycin, Neomycin	Gram negative bacteria	Binding to the bacterial 30S or 50S ribosomal, inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA, leaving the bacterium unable to synthesize proteins vital to its growth
<b>Cephalosporin's</b>			
1st Generation	Cefazolin, Cefalexin, Cefalotin	Gram positive bacteria	Same mode of action as other beta-lactam antibiotics: disrupt the synthesis of the peptidoglycan layer of bacterial cell walls
2nd Generation	Cefuroxime, Cefoxitin, Cefoxitin	Less Gram positive, improved Gram negative	
3rd Generation	Cefoperazone, Cefotaxime, Ceftriaxone	Mainly Gram negative bacteria except Pseudomona	
4th Generation	Cefepime	Covers Pseudomonas	
5th Generation	Ceftaroline, fosamil, Ceftobiprole	Used to treat MRSA	

Glycopeptides	Vancomycin, Teicoplanin	Gram positive bacteria	Inhibiting peptidoglycan synthesis
Macrolides	Erythromycin, Azithromycin, Clarithromycin	Mostly Gram positive	Inhibition of bacterial protein biosynthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl tRNA.
Penicillin	Amoxicillin, Ampicillin, Methicillin, Penicillin	Mostly gram positive	Same mode of action as other beta-lactam antibiotics: disrupt the synthesis of the peptidoglycan layer of bacterial cell walls
Quinolones	Ciprofloxacin, Nalidixic acid, Norfloxacin, Ofloxacin	Gram positive and negatives	Inhibit the bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription
Sulphonamides	Sulphamethizole, Trimethoprim/Sul famethoxazole	Gram negative	Inhibit folate synthesis
Tetracyclines	Tetracycline, Oxytetracycline	Gram positive and negatives	Inhibiting the binding of aminoacyl tRNA to the mRNA-ribosome complex. They do so mainly by binding to the 30S ribosomal subunit in the mRNA translation complex.
Chloramphenicol	Chloramphenicol	Gram positive and negatives	Inhibits bacterial protein synthesis by binding to the 50S subunit of the ribosome

Source of Table 2 which reports on common use and mechanism(s) of action of antibiotic groups: Adzitey (2015)

## 2.5 Use of antibiotics in animal food chains

Antibiotics have been used in livestock production for several purposes, such as: animal disease treatment, animal disease prevention, and feed efficiency (Jank *et al.*, 2015). Antibiotics are also commonly used in sub-therapeutic amounts for prophylaxis to prevent an infection within the flock or herd Mensah *et al.* (2014). An increase in the use of antibiotics has caused the bacteria to be increasingly resistant ensuring their survival against the load of antibiotic agents which are continuously administered (Fomnya *et al.*, 2021). Diseases like mastitis are one of many other reasons why antimicrobial use has gained attention in dairy farming practices (Pyörälä, 2009).

## 2.6 Commonly used antibiotics

Among antimicrobial (antibiotic) drugs, oxytetracycline (OTC) and sulphamethazine (SMZ) are frequently used in veterinary management of livestock diseases in Zambia (Mainda *et al.*, 2015). OTC has been successfully used worldwide in both veterinary and aquaculture fields because it is cheap and has broad antimicrobial spectrum against both gram positive and gram-negative bacteria (Chopra & Roberts, 2001).

They are several antibiotics such as; tetracyclines (chlortetracycline, oxytetracycline, tetracycline), penicillin's (amoxicillin, ampicillin), aminoglycosides (gentamycin, neomycin, streptomycin), macrolides (erythromycin, tilmicosin, tylosin), cephalosporin's third generation (ceftiofur), glycopeptides (avoparcin, vancomycin), quinolones or fluoroquinolones (sarafloxacin, enrofloxacin), streptogramins (virginiamycin, quinupristin-dalfopristin), sulphonamides (sulphadimethoxine, sulphamethazine, sulphisoxazole), polypeptides (bacitracin) and lincosamides (lincomycin), are among the most used in agriculture (Adzitey, 2015; Manyi-Loh *et al.*, 2018; Ullah & Ali, 2017).

Mainda (2016) reported that tetracyclines and penicillins are the most commonly used antibiotics among the dairy farmers in Zambia. The high resistance to tetracyclines, sulphonamides and beta-lactam antibiotics in Zambia could be attributed to the misuse in both livestock and humans (Muonga, 2021). They are commonly used because of cheap price for most livestock farmers (Mainda *et al.*, 2015). Phiri *et al.* (2020) revealed high levels AMR of *Escherichia Coli* towards tetracycline due to its extensive use in livestock production systems in Zambia. Nchima *et al.* (2017) found a significant prevalence of OTC residues, along with SMZ residues, in beef samples within Zambia.

## 2.7 Epidemiology of antibiotic residues in milk

### 2.7.1 Definition of antibiotics and origin

Antibiotics are substances that can either kill or inhibit the growth of bacteria (Walsh, 2022). Those antibiotics that kill the microorganisms are called bactericidal while those that inhibit the growth of microorganisms are called bacteriostatic (Walsh, 2022). In the efforts to address the challenges of infectious diseases, but also to find a substance that could kill, inhibit or at least slow down the growth of such disease-causing bacteria, led to the revolutionary discovery of the antibacterial agent “penicillin” in 1928 from *Penicillium notatum* by Sir Alexander Fleming (Kumavath, 2017).

Bacteria are simple one-celled organism, which were first identified in 1670s by Van Leeuwenhoek (Kumavath, 2017). According to Fomnya *et al.* (2021) following the introduction of penicillin in the

1940s, antibiotics use has become one of the cornerstones of modern medicine. The discovery of antibiotic agents unlocked the field of microbial natural products and thus new agents were continually added such as daptomycin, tigecycline and linezolid (Ullah & Ali, 2017).

### **2.7.2 Occurrence of antibiotic residues and factors associated**

Globally it is anticipated that consumption of antibiotics is projected to rise by 67% between 2010 and 2030 due to the need for increased livestock production which is also linked to increased application of antibiotics (Patel *et al.*, 2020). This is somewhat related to an increased demand for animal protein especially in Asia (Patel *et al.*, 2020). Presence of antibiotic residue in ASF is an important problem in most countries (Chowdhury *et al.*, 2015). Among the different types of antimicrobials, antibiotics are extensively used in medical and veterinary practices. Practically, 80% of globally manufactured antibiotics are used in the animal production (Almashhadany, 2020).

Mudenda *et al.* (2022) described that inappropriate dispensing and use of antibiotics in animals during treatments, prophylaxis and growth promotion has precipitated the deposition of antibiotic residues in ASF like milk, meat, poultry, fish and eggs. Misuse and overuse of drugs in the treatment of lactating dairy animals can result in the contamination of milk with levels of drug residues in excess of established MRL's thereby rendering the milk unsuitable for human consumption and food product manufacturing.

Indiscriminate use and vast antibiotic application in the livestock sector can result in deposition of antibiotic residues in ASF and these antibiotic residues can adversely cause a threat to human health (Hossain *et al.*, 2023). A study conducted by Omairi *et al.* (2022), illustrates that there is very little information known about potential effective methods on how to reduce the detrimental outcome of antibiotic misuse in developing countries, due to the lack of structured research and regulations among others.

Additionally, high disease burden and indiscriminate uses of antibiotics in developing countries inclusive of Africa, had led to deposition of antibiotics in ASF. This is due to little or no capacity for; residue testing as a surveillance protocol, strict regulations with regards to the use of antimicrobial drugs, knowledge and effective dairy principles, purchase of antibiotics and enforcement of antibiotic withdrawal periods (Chowdhury *et al.*, 2015; Mohamed *et al.*, 2020). According to Booth *et al.* (2020) contamination of milk with antibiotics was reported to be as a result of intra-mammary infusions of antibiotics (92%), injections (6%), and other causes 2%.

ARs occur as a result of improper use of a licensed drug or through the illegal use of an unlicensed substance. The residual antimicrobials in food constitute a risk to human health, but poor knowledge is available about the significance of contaminated ASF in developing countries (Rakotoharinome *et al.*, 2014). Shukla *et al.* (2011) reported that while most countries banned antibiotics known to be toxic for human, e.g., chloramphenicol or avoparcin, from animal food production, these banned drugs are still reported to be used in developing countries. Most lay farm workers often make their own decisions and administer treatment themselves (Raymond *et al.*, 2006).

Availability of knowledge and good management practices among farmers and consumers plays a vital role in addressing this public health challenge of ARs. However, misuse of antimicrobial drugs in farms and residues in ASF becomes a major issue for veterinary sector and for public health services in Africa (Ibrahim *et al.*, 2012; Kang'ethe *et al.*, 2005). The risk of contamination of farm milk with antibiotic residues is higher if inappropriate management practices are applied (Layada *et al.*, 2016).

In the recent past, milk producers have faced a growing demand and pressure for milk and its producers therefore, resorting to boost milk production among their dairy cattle. This has subsequently led to an increased use of antibiotics to address the challenges of infections (Shitandi & Kihumbu, 2004). In many low and middle income countries, the use of antimicrobials (antibiotics) for treatment of disease still remains undocumented and unregulated (Gilbert *et al.*, 2020).

Additionally, in an event that dairy farmers milk their animals before the completion of the prescribed antimicrobial withdrawal period, antibiotic residues can be found in the milk above MRL. A study conducted by Kunda (2015) in Lusaka province of Zambia reported a prevalence of 30.1% positive smallholder dairy farms. The presence of antibiotic residues in milk above MRL set by international authorities like Codex Alimentarius Commission (CAC) constitutes a serious global public health threat such as; development and sustenance of antibiotic-resistant bacteria and other effects like allergic reactions and cancer (Cinquina *et al.*, 2003).

### 2.7.3 Antibiotic consumption and use in domestic animal source food production

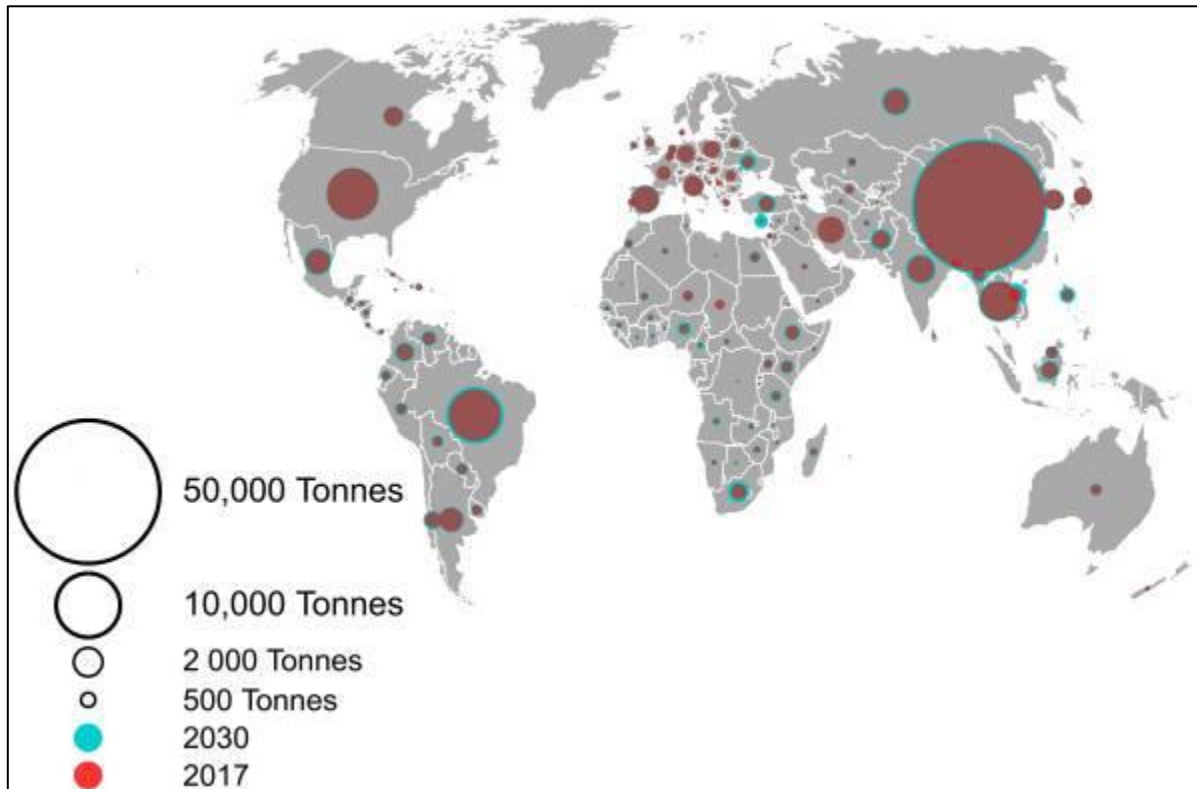


Figure 1. Consumption levels of antibiotics for all domesticated animal species

Source for Figure 1 which reports on antibiotic consumption and use in domestic ASF production: Kimera *et al.* (2020)

Above in Fig 1, Asia exhibits the highest antibiotic consumption globally because it is the most populous continent, while South Africa leads in antibiotic consumption data within Africa.

### 2.7.4 Public health significance

The presence of antibiotics residues in ASF above the MRLs has been recognized worldwide by various public authorities due to public health threats (Kempe *et al.*, 2000). Public health implications of AMR in ASF likely include; toxicity of bone marrow, destruction of microflora in human intestinal microflora, allergic reactions, cancer and development and sustenance of antibiotic-resistant bacteria and antibiotic resistance genes (Cinquina *et al.*, 2003).

Antibiotic residues in ASF produces potential threat to direct toxicity in humans such as cancers, allergic reactions and low levels of antibiotic exposure results in alteration of microflora, and the possible development of AMR which cause failure of antibiotic therapy in clinical situations

(Ahaduzzaman, 2014; Hassan, 2014). According to Phiri *et al.* (2020) the overall implication of AMR in Zambia is that these antibiotic resistant bacteria and genes may be transmitted to humans and animals where they may end up causing treatment failure and thereby, leading to increased morbidity and mortality.

Over the years, AMR has reduced the efficacy of last resort antibiotics, which in turn led to prolonged hospital admission stay, increase in severity of infectious diseases and reductions in treatment options (Manyi-Loh *et al.*, 2018; Page & Gautier, 2012). The application of antibiotics in food-producing animals mainly in developing countries where disease prevalence and antibiotic usage is high has greatly threatened the benefits of antibiotic stewardship (Kempe *et al.*, 2000). The presence of ARs in ASF led to developing antimicrobial-resistant bacteria and releasing them into the environment and on different products of animal origin which are used as food for humans (Landers *et al.*, 2012).

In 2019, a report from WHO (2019) declared AMR as one of the top ten global public health threats. Globally 4.95 million people who died in 2019 suffered from drug-resistant infections. AMR directly caused 1.27 million of those deaths. Around 2019 in Zambia, there were 3,700 deaths attributable to AMR and 15,600 deaths associated with AMR (WHO, 2019). AMR represents a global challenge. In addition, a report from EFSA (2011), mentioned that EU and the Member States identified AMR as one of the top three priority health threats. Zambia has the 14<sup>th</sup> highest age-standardized mortality rate per 100,000 populations associated with AMR across 204 countries (WHO, 2021).

### **2.7.5 Interventions to reduce antimicrobial residues (antibiotics) in milk**

The overall strategy for the prevention of ARs in milk and other ASFs is based on procedures and drug use at the farm level (Codex, 2001). The use of antibiotics is an essential tool in treating bacterial infections, however responsible use is encouraged to help preserve their effectiveness for the future (Hossain *et al.*, 2023). The prevention of ARs in the milk supply is the responsibility of both dairy industry and the regulatory authorities such as veterinary institutions (Codex, 2001).

This responsibility rests primarily on the dairy producer, who controls the hygienic conditions of the milk production facility and, the environment of the dairy animals and administers drugs to dairy animals. The veterinarian who controls the selection, administration and the use conditions of drugs administered to dairy animals shares this responsibility (Codex, 2001). Majority of countries globally, regulate drugs use in ASF and set up MRLs to ensure food safety and promote public health (Chowdhury *et al.*, 2015).

Surveillance and monitoring of ARs in ASF is of great importance to assure food safety (Barros *et al.*, 2023). Considering an alert to the crisis of AMR and its global public health threat, in 2015 the World Health Assembly adopted a GAP on AMR (WHO, 2019). The formulation GAP on AMR is the world's blueprint for tackling the emergence and spread of AMR, which threatens many of the global Sustainable Development Goals (SDGs) on health, food security, environmental well-being and socioeconomic development (WHO, 2019).

In order to ensure public safety, worldwide regulatory authorities have set MRLs for several antimicrobial drugs (Zeina *et al.*, 2013). There is need for adherence to good practices of antimicrobial usage in both human and veterinary medicine. Due to global public health impact of AMR, antimicrobial stewardship strategies must be tailored specifically from the ground level by ensuring that routine surveillance of antibiotic residues and drug withdrawal period are monitored. AMR should be addressed via “One Health” strategy (Kapona, 2017). There is need for adequate control measures to be implemented for prevention of ARs from entering into the food chains (Layada *et al.*, 2016).

The approach of “One Health” is a collaborative effort of multiple disciplines working locally, nationally, and globally to attain one common goal thereby, achieving optimal health for people, animals and our environment (Mackenzie & Jeggo, 2019). Kapona (2017) described that Zambia developed a multi-sectorial national action plan using a “One Health” approach with required strategies for combating AMR in humans, animal and environment. Nchima *et al.* (2017) recommended for enactment of friendly legislation, surveillance plan to reduce ARs in ASF. Nchima *et al.* (2017), reported that antibiotic residues in ASF need to follow the withdrawal period.

## **2.8 Economic significance**

The presence of ARs above MRLs in milk tends to deteriorate the dairy industry by inhibiting the cultivation of starters during cheese production and other fermented dairy products and decreases the production of desired acids and flavours (Sachi *et al.*, 2019). Economic burden per year due to healthcare costs and productivity losses caused by AMR is estimated in EU at EUR 1.5 billion and \$55 billion in the U.S (Mobarki *et al.*, 2019; WHO, 2019). Losses can also be due to a state of prolonged hospital admission due to reduced efficacy of last resort antibiotics and increased severity of infectious diseases and reductions in treatment options (Manyi-Loh *et al.*, 2018).

## 2.9 Global legal framework of (MRLs) of antibiotics in foods of animal source

In order to minimise human exposure to ARs the organisation's like CAC established Codex guidelines as regulatory program for control of ARs in foods Codex (2001), European commission (EC) European Council (2011) and the Food and Drug Administration FDA (2022) established MRLs limits for pharmacologically active substances. The Global legal framework offers clear definitions of terms used in antibiotics in ASF as indicated below:

**Residue:** defined by EU as a “pharmacologically active substances which can either be active principles, recipients, or degradation products and their metabolites which are retained in ASF to which the veterinary drug in question has been administered as defined by (EFSA, 2011).

**Antibiotic residues:** these are residues that result from administered parent antibiotics or their metabolites that become deposited and retained in animal tissue and matrix intended for human consumption, where the concentration is beyond the MRLs for a certain period of time (Nisha, 2008).

**Maximum Residue Limit (MRL):** MRLs means the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be accepted by the community to be legally permitted or recognized as acceptable in or on a food (EFSA, 2011). According to Lee *et al.* (2001) the MRLs are defined as the maximum allowable level of a chemical in feed or food at a specified time of slaughter or harvesting, processing, storage and marketing up to the time of consumption by animal or human. The MRLs for certain antibiotics in milk, as set by the Codex CAC in July 2009 during 32<sup>nd</sup> session (CAC, 2011) and the EC in Council Regulation 2377/90/EC (Ancuceanu, 2003), are listed below;

Table 3: CAC and EU MRLs of different veterinary antibiotics residues in cattle milk.

Type of antibiotic agent	CAC MRLs (µg/L)	Year of adoption by CAC	Type of antibiotic agent	EU MRLs (µg/kg)
Benzyl-penicillin/procaine	4	1999	Benzyl penicillin	4
Benzyl-penicillin				
Chlortetracycline/oxytetracycline/tetracycline	100	2003	Ampicillin	4
Dihydrostreptomycin/streptomycin	200	2003	Amoxicillin	4

Gentamycin	200	2001	Tetracycline	100
Lincomycin	150	2003	Oxytetracycline	100
Neomycin	1500	2005	Chlortetracycline	100
Sulfadimidine	25	1995	Streptomycin	200
Tylosin	100	2009	Dihydrostreptomycin	200
			Gentamicin	200
			Neomycin	1500
			Spiramycin	200
			Tylosin	100
			Erythromycin	40
			Colistin	50
			Ceftiofur	100

Source of Table 3 which indicates CAC and EU MRLs of different veterinary antibiotics residues in cattle milk : Ancuceanu (2003); CAC (2011)

### **2.10 Legal framework of MRLs of ARs in Zambia**

Zambia has a dedicated institution for product standardization known as the Zambia Bureau of Standards (ZABS). ZABS specializes in standardization, food safety, creation of standards, quality control, quality assurance, and conducting quality inspections for imports and exports. It also focuses on certifying products and eliminating technical barriers to trade. The MRLs for all antimicrobials in ASF are established by ZABS, referencing Codex standards to ensure food safety. Recently, Zambia has made strides in addressing food safety issues by enacting the Food Safety Act No. 7 of 2019 of the National Assembly of Zambia (2019), which prohibits the sale of any food that fails to meet safety and quality standards, thereby safeguarding public health.

### **2.11 Antibiotic extraction techniques**

There are several techniques that have been described to detect ARs in milk and its products. These available screening tests include; microbial inhibition assays, rapid test kits, immunoassays and biosensors (Wang, 2010). They are mostly preferred due to low costing, simplicity, large number of samples per kit and possibility of automation (Toldrá & Reig, 2011). In practice, antibiotic residue screening is primarily performed using microbiological screening methods, because of their high cost-

effectiveness compared to physical–chemical detection. In comparison, a liquid chromatography technique is more sensitive than microbial inhibition assays-Delvotest (Layada *et al.*, 2016).

The immunological methods such as Enzyme Linked Immunosorbent Assays (ELISA) are commonly used and are typically very specific and aid in the detection of ARs from ASF, operate on the basis of antigen-antibody interactions (Johnson, 2013). Charm II system (radio receptor assay technique) was developed by Charm Sciences Inc. and is one of the rapid screenings and 100% group specific techniques for detection of ARs classes such as sulphonamides, beta-lactams, tetracyclines, chloramphenicol, quinolones, macrolides and aminoglycosides in various ASF including; fish, poultry, meat, eggs, honey and milk (Kwon *et al.*, 2011a; Science Inc, 2024).

Furthermore, chromatographic techniques such as liquid chromatography is a confirmatory method stated by Wang (2010), due to its superior specificity, quantify the analyte and provide structure elucidation when being coupled with mass spectrometers hence widely used (Nicolich *et al.*, 2006). According to a study conducted by Petkovska *et al.* (2006), chromatographic techniques namely; thin layer chromatography (TLC), and high performance liquid chromatography (HPLC), and capillary electrophoresis (CE), have been developed to replace microbiological assays.

### 3. CONCEPTUAL FRAMEWORK

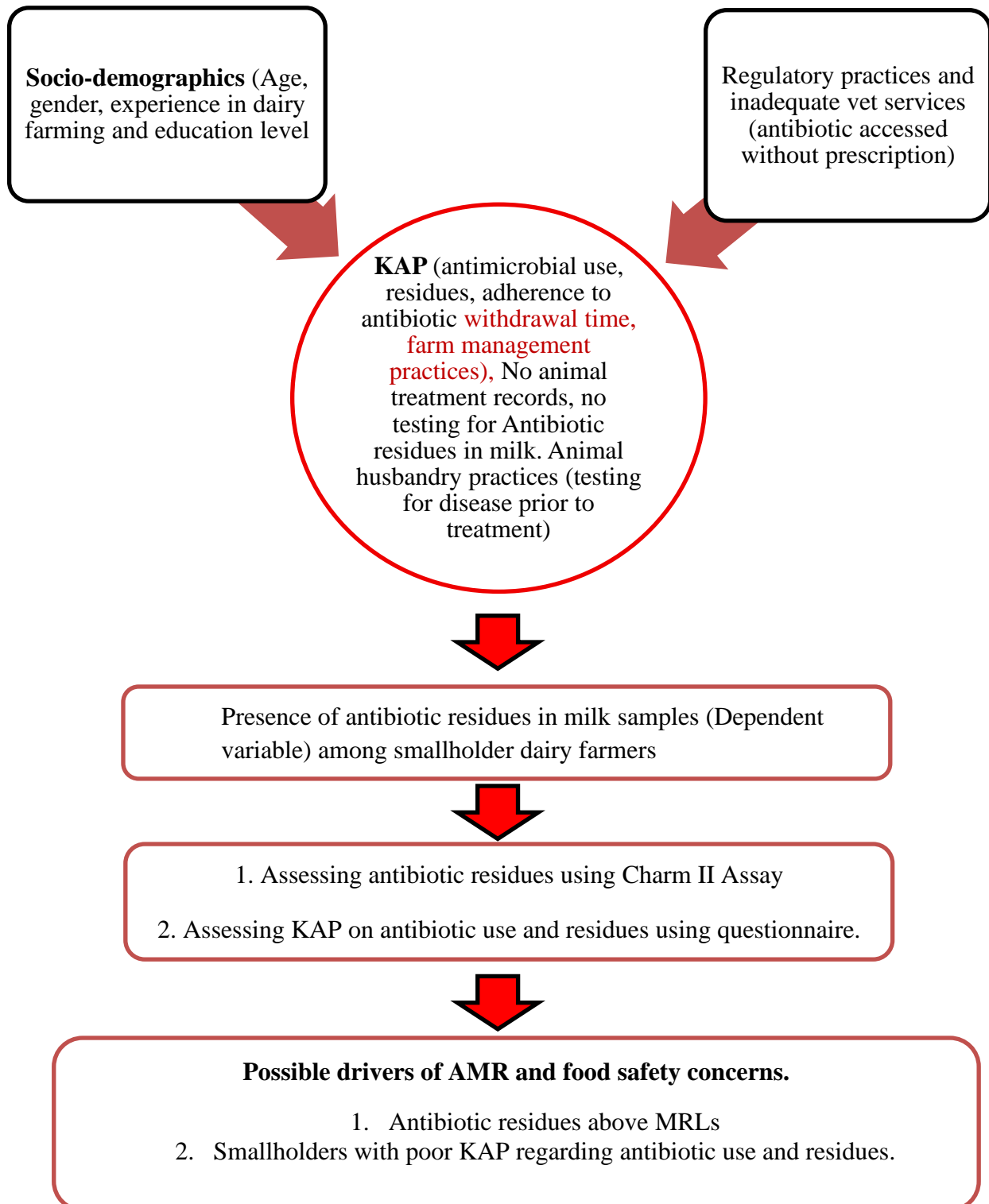


Figure 2. Conceptual framework of antibiotic residues in milk

## 4. MATERIALS AND METHODS

### 4.1 Location of the study

The study was conducted in Kasama and Mbala districts of Northern Province, Zambia. Zambia is located in Southern central Africa. Mbala is situated at 8°50'S latitude and 31°28'E longitude, south of the equator. The district is located in the north-western part of the province, adjacent to Tanzania to the north, and it borders Senga Hill, Nakonde, Kasama, and Mpulungu districts (Figure 3). Mbala covers an area of approximately 10,832km<sup>2</sup>. Its altitude ranges from 400m to 2,067m above sea level. The region experiences a temperate climate characterized by mild temperatures and distinct wet and dry seasons, with an average annual rainfall of 1, 200mm.

On the other hand, Kasama is located at 10°10'S latitude and 31°15'E longitude. Kasama district lies centrally in the Northern Province, about 167 km south of Mbala (Figure 3). The district spans roughly 10,788km<sup>2</sup> with an average altitude of 1,300m. The region experiences a humid subtropical climate, closely similar to tropical savannah climate, characterized by distinct wet and dry seasons, with an average annual rainfall of 1,200 mm.

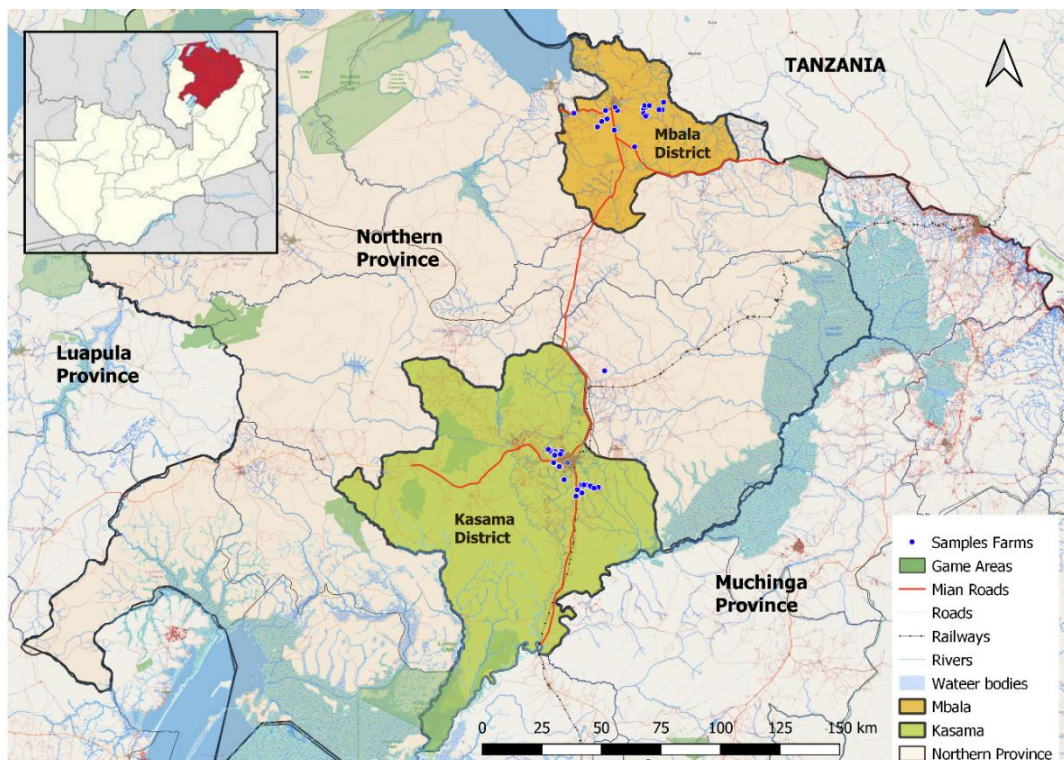


Figure 3. Map of Zambia and location of Kasama and Mbala study areas. The map was generated in QGIS Version 3.38.0).

## **4.2 Description of the subject**

To conduct this research, raw cow (bovine) milk samples were collected among smallholder dairy farmers to detect antibiotic residues and assess their level knowledge, attitude and knowledge about antibiotic use and residues.

### **4.2.1 Characterization and origin**

Kasama and Mbala were purposively selected because they are the only districts in Northern Province, Zambia, with the highest number of active smallholder dairy producers at the time of this study. Dairy production in these districts primarily occurs on a traditional or small scale. Most smallholder dairy producers were members of established dairy cooperatives and typically sold milk on their farms to consumers or at times supplied to local markets. Smallholder dairy producers were unable to supply milk to their local MCC due to their non-operational status. Northern Province was chosen for this study because no similar research had been conducted previously.

Additionally, smallholder dairy producers did not supply milk to commercial off-takers, who typically require compliance with MRLs for ARs in milk. This created a significant risk that farmers may not adhere to withdrawal periods for antibiotic residues in the milk they produced. The province also lacked commercial off-takers who would likely monitor antibiotic residues at collection points during the supply of milk from smallholder dairy producers. Smallholder dairy production was described as village or range-land production with minimal or no biosecurity and products (milk) sold or consumed locally or sold to the local processors with production capacity of 5-50 cattle herd size.

### **4.2.2 Criteria of Inclusion/exclusion**

The study randomly selected smallholder dairy producers in Kasama and Mbala districts. The inclusion criteria for milk sampling employed any cow producing milk at the time of study. All dairy cows (bovine) not producing milk at the time of study were excluded from the study. The inclusion criteria for a questionnaire included both smallholder dairy producer's actively producing milk and those whose dairy animals were on dry period at the time of study. However, smallholder dairy producers who were not producing milk and their animals were not on dry period at the time of the study were excluded from the study. Milk samples and questionnaires were collected at each farm. When the initially selected smallholder dairy farmers were unavailable for the sampling/questionnaire phases, they were substituted by others.

### **4.2.3 Study population**

A sampling frame was derived from the district stock registers issued by the District Livestock Production and Extension office in both districts.

#### **4.2.3.1 Study population for milk samples**

There was a total of 103 individual dairy cattle producing milk in both Kasama and Mbala district (43 and 60 respectively) at the time of this study.

#### **4.2.3.2 Study population for questionnaire survey**

There was a total of 109 smallholder dairy producers across both districts. In Kasama, there was 63 dairy producers, with 41 actively producing milk and 22 having animals in a dry period. In Mbala district, there were 46 smallholder dairy producers, of whom 21 were actively producing milk while 25 had their cattle in a dry period.

### **4.2.4 Limitations**

This study only focused on qualitative analysis of antibiotic residues in milk samples collected from smallholder dairy producers within Mbala and Kasama districts of Northern Province, Zambia. This study did not include other livestock producers at the time of study. This research did not cover the entire province due to limited resources and time. The spatial distribution of smallholder dairy farmers across the two districts extended the milk sample and questionnaire survey collection period. Additionally, social gatherings, such as funerals, affected the availability of smallholder dairy farmers, mainly for the questionnaire survey.

## **4.3 Research design**

A cross-sectional study was conducted among randomly selected smallholder dairy producers in the two districts between May to June, 2025. This research was applied in nature because it aimed to assess the presence of various classes of antibiotic residues in cow (bovine) milk collected from smallholder dairy producers. The study further assessed the farmers level of knowledge, attitude and practice on antibiotic use and presence of residues in milk following limited data on antibiotic use, residues and surveillance protocols of antibiotic residues in food of animal origin in Zambia (Mainda *et al.*, 2015).

### **4.3.1 Sample size calculation**

A 2-stage or double sampling method was employed which utilised Proportional Probability to Size (PPS) as a self-weighting method to avoid bias. The sample size per district was estimated with the **EpiTools epidemiological calculators** (<https://epitools.ausvet.com.au/?page=home>) using the

standard sample size formula for estimating a single proportion from simple random sampling described by (Thrusfield & Christley, 2018).

Sample size is calculated using the formula:  $n = (Z^2 \times P \times (1 - P))/e^2$ ..... (1)

Where:

- **n**= Required sample size
- **Z** = value from standard normal distribution corresponding to desired confidence level (Z=1.96 for 95% CI)
- **P** = expected true proportion or estimated prevalence of antibiotic residues in raw milk from smallholder farmers (from previous studies or assumed, if no previous data exist use 50% or 0.5 which yields a maximum sample size)
- **e** = desired precision (half desired CI width).

The targeted sample size of milk samples was calculated to be 91 from a target population of 103 dairy cattle. This included; 39 samples in Kasama and 52 samples in Mbala @ 95% confidence level. In addition, the sample size for a questionnaire survey was calculated to be 97 from a total target population of 109 smallholder dairy producers (55 in Kasama and 42 in Mbala district).

#### **4.3.2 Milk sample collection**

Milk samples were collected from each randomly selected farm. Thereafter, at each farm, a milk sample was collected from every randomly selected individual dairy cow. Each cow (bovine) functioned as an independent epidemiological unit. Approximately 50-100ml of milk samples were collected from each dairy cow and placed into 50ml sterile plain bottle (NVDRMP, 2017). Samples were collected in duplicate of 50ml making a total of 100ml per animal because only 50ml were available.

All bottles were properly labelled with an allocated animal ID and date. After collection milk samples were placed in the cool box containing frozen dry ice packs and transferred to the district laboratory (within 4 hours), where it was stored in the freezer (Mohamed *et al.*, 2020). After completion of sample collection, all frozen samples were stored in a cooler box with frozen dry ice and sealed tightly before transported to the Central Veterinary Research Institute (CVRI) in Lusaka, Zambia, five days after collection. At CVRI, samples were stored in a freezer set at -20°C (Chowdhury *et al.*, 2015). Analysis was done using Charm II receptor assay protocols (Science Inc, 2024b).

### **4.3.3 Questionnaire survey using KoboToolbox**

To allow document validation, the questionnaire was reviewed by statistical and epidemiological experts from the University of Eduardo Mondlane. This allowed for pre-validation process to facilitate; simplicity, relevance, accuracy, clarity and logic as described by Mudenda *et al.* (2023). A semi-structured questionnaire was adopted according to a study by Mudenda *et al.* (2023). A questionnaire survey posted on KoboToolbox (v2024.2.4) open-source data collection platform was randomly administered to 101 selected smallholder dairy producers actively producing milk and/or only own dairy cows on dry period at the time of study.

Personal interviews of smallholder dairy producers were done to assess their level of knowledge, attitude and practices regarding antibiotic use and residues (Mohamed *et al.*, 2020). Face-to-face interviews were conducted by the principal investigator and two research assistants (local veterinary assistant) from each study area. The questionnaire survey was conducted for 20-30 minutes using three languages namely; English, Bemba and Mambwe. This study recruited five (5) questionnaires to be used during a pilot study within Sika village of Mbala district.

The piloting study was done in association with Eduardo Mondlane University Faculty of Veterinary Science to help incorporate minor modifications following suggestions from respondents. The respondents in the pilot study (5 respondents) were excluded from the final analysis. The questionnaire survey was divided into three (3) sections, namely, Section A for demographic data, Section B for Epidemiological data and Section C for assessment of levels of KAP on antibiotic use and residues.

### **4.3.4 Determination of antibiotic residues using Charm II system analysis**

Milk samples were prepared and screened using a Charm II system (Science Inc, 2011) which was installed at CVRI. This study utilized the Charm® II competitive receptor assay which is a competitive rapid receptor assay used to screen for the presence of antibiotic class residues in ASF at EU/MRLs (Science Inc., 2010). The milk samples were screened only for five (5) classes of antibiotic residues namely; beta-lactams, tetracyclines, sulphonamides, aminoglycosides and macrolides.

Before each analysis, a specified number of frozen samples were placed in a rack at room temperature for approximately 3 hours to allow thawing and then were gently swirled up and down 20 times for 20 seconds allowing thorough mixing. The protocol was slightly modified during validation of critical control point set-up by including only one (1) zero/negative control instead of three (3) zero control

standards for sulphonamides, tetracyclines, aminoglycosides and beta-lactams classes while macrolides used one (1) positive control standard. This was due to the limited availability of tracer and binder tablets. Despite these modifications during validation process, the analysis protocol for all antibiotic classes remained unchanged

#### **4.3.4.1 Charm® II Beta-lactam Test for Cow Milk at MRL**

##### **4.3.4.1.1 Zero control standard preparation**

A Zero control standard milk powder was included in the kit certified organic whole milk powder to be antimicrobial drug free. The milk was reconstituted with 100 ml of 40°C water as indicated on the label and was shaken well (until all clumps were broken up). The milk solution was then cooled by refrigeration before use and stored other dry standards in the refrigerator. The reconstituted standard was held under refrigeration for up to 72 hours (Science Inc, 2010).

##### **4.3.4.1.2 Positive control standard preparation**

A multi-antimicrobial standard (containing  $4.00 \pm 0.15$  ppb penicillin G when reconstituted) was supplied with the kit manufactured from USP reference standards and was used as a positive standard. This positive standard was reconstituted with 50 ml negative control or zero control standard and the mixture was shaken well and allowed to stand refrigerated for 15 minutes. This reconstituted positive control mixture was thoroughly mixed before each use. The dry positive control standard that remained was kept in the refrigerator while the reconstituted positive control standard was held in the refrigerator for a period of 48 hours (Science Inc, 2010).

##### **4.3.4.1.3 Beta-lactam test procedure for Sample or control**

A green tablet was added in the empty test tube and 300 µl water was added and this was mixed using a vortex (model MX-5) for a minimum of 10 seconds in order to completely break up tablet. To this mixture a 5.0 ml of the sample or control was added. Then immediately mixed using a vortex (model MX-5) for 10 seconds. This mixture was then incubated at 65°C for 2 minutes and a yellow tablet was added which contained less than 0.2 kilobecquerels (kBq) of [<sup>14</sup>C]-penicillin G) and immediately mixed by swirling milk up and down 10 times for 10 seconds. This mixture was incubated (Inctronic 2) at 65°C for 2 minutes and centrifuged (Rotofix 32A) for 3 minutes at 3400 rpm.

The mixture was removed from centrifuge, poured off milk and removed the fat ring and the test tube was wiped dry with cotton swab without disturbing the pellet. Then 300 µl water was added and

thoroughly mixed using a vortex (model MX-5) to break up pellet and ensured it is suspended in water before adding scintillation fluid. Then 3.0 ml of scintillation fluid was added and capped the test tube and mixed using a vortex (model MX-5) until mixture had a uniform cloudy appearance. This mixture was then placed in the analyzer (Charm II 6600 counter version 3.12) for 60 seconds and read count per minute (cpm) on [14C] channel. In an event the cpm was greater than and within 50 cpm of the control it was recounted in the analyser (Science Inc, 2010).

#### **4.3.4.1.4 Control point set-up**

A total of six replicates of the positive control standards were run in the analyzer and determined their average. To this calculated average a 15% was added and this was considered as the control point. The control point was valid if all six determinations were within 15% of the positive average. Cases of single deviants were retested in the counter analyzer and another averaging was done while if they were more than one deviant values, then control point determination was repeated (Science Inc, 2010).

### **4.3.4.2 Charm® II Sulphonamides Test for Cow Milk at MRL**

#### **4.3.4.2.1 Zero control standard preparation**

A zero-control standard supplied with the kit (certified organic whole milk powder to be antimicrobial drug free) was used as negative control standard. A zero-control standard was reconstituted with a 100 ml of 40°C water as indicated on label and was nicely shaken until all clumps were broken up. The remaining zero control standard was kept refrigerated while the reconstituted mixture was stored in the refrigerator set at 0 to 4.4°C before use and the mixture was allowed to stay for up to 72 hours (Science Inc, 2011).

#### **4.3.4.2.2 Positive control standard preparation**

A 100 ml of  $10.0 \pm 0.4$  ppb sulfamethazine standard which was supplied with the kit and manufactured from USP reference standard was reconstituted with a 100 ml of negative control (zero control standard). The mixture was then nicely shaken and allowed to stand in the refrigerator for 15 minutes before use. The dry positive control standard that remained was kept in the refrigerator while the reconstituted sulfamethazine positive standard was held in the refrigerator at 0 to 4.4°C for a period of 48 hours (Science Inc, 2011).

#### **4.3.4.2.3 Sulphonamides test procedure for Sample or control**

A white tablet was added to an empty test tube and a 300 µl of water was also added and mixed for 10 seconds using a vortex (model MX-5) until the tablet was completely broken. During addition of each sample to the test tube, a new tip was used to avoid contamination. A 5.0 ml of sample or control

standard at a temperature of 0 to 7°C was then added to this mixture and thoroughly mixed for 10 seconds. Thereafter, a pink tablet (containing 3.0 kilobecquerels (kBq) of [3H]-sulfamethazine) was added and was immediately mixed with vortex mixer (model MX-5) or swirling milk up and down 15 times for 15 seconds.

The mixture was then incubated (Inctronic 2) at 85°C for 3 minutes and centrifuged (Rotofix 32A) for 3 minutes at 3400 rpm. After centrifuging, the milk was then poured off and the fat ring was removed and the test tube was wiped dry with swabs without disturbing the pellet. A 300 µl of water was added and mixed using a vortex mixer (model MX-5) to break up pellet by ensuring it was suspended in water. Then a 3.0 ml of scintillation fluid was added, capped the test tube and mixed using vortex mixer until a uniform cloudy appearance. A test tube with the mixture was then placed into the analyzer (Charm II 6600 counter version 3.12) for 60 seconds to allow counting and read cpm on [3H] channel (Science Inc, 2011).

#### **4.3.4.2.4 Control point set-up**

A total of 6 replicates of positive control standards were run in the analyzer and averaged the 6 cpm results. In an event any of the six determinations deviated by more than 15% from the calculated average, then that determination was replaced and then another averaging was done. After averaging, a 24% of the final average was added to calculate the control point (Science Inc, 2011).

### **4.3.4.3 Charm® II Tetracycline Test for Cow Milk at MRL**

#### **4.3.4.3.1 Zero control standard preparation**

A zero-control standard supplied with the kit (certified organic whole milk powder to be antimicrobial drug free) was used as negative control standard. A zero-control standard was reconstituted with a 100 ml of 40°C water as indicated on the label. The remaining zero control standard was refrigerated while the reconstituted mixture was nicely shaken until all clumps were broken up and stored in the refrigerator set at 0 to 4.4°C before use and was kept for a period of 72 hours (Science Inc, 2024d).

#### **4.3.4.3.2 Positive control standard**

A multi-antimicrobial standard (containing 30 ppb oxytetracycline when reconstituted) which was supplied with the kit (manufactured from USP reference standards) was reconstituted with a 100 ml of negative control or zero control standard. This mixture was nicely shaken to be thoroughly mixed and was allowed to stand in the refrigerator for 15 minutes before use. The dry positive control

standard that remained was kept in the refrigerator while the reconstituted positive control standard was held refrigerator for a period of 48 hours (Science Inc, 2024d).

#### **4.3.4.3.3 Tetracycline Test – Procedure**

All samples or reconstituted standards were nicely mixed before use testing for 10 seconds. Thereafter, a white tablet was added to an empty test tube and a 300 µl of water was added and mixed using a vortex mixer (model MX-5) for 10 seconds to completely break up tablet. To this mixture a 5.0 ml of sample or control was added while using a new pipet tip for each sample to avoid contamination. Thereafter, an orange tablet (containing 1.85 kilobecquerels (kBq) of [3H]-tetracycline) was added and immediately mixed using a vortex mixer (model MX-5) or by swirling milk up and down 15 times for 20 seconds.

Thereafter, the mixture in the test tube was then incubated (Inctronic 2) at 35°C for 3 minutes and centrifuged (Rotofix 32A) for 5 minutes at 3400 rpm. This mixture was then immediately removed from centrifuge and poured off milk and broke the fat ring with cotton swab while the test tubes were inverted. Afterwards, the test tube was wiped dry with cotton swabs without disturbing the pellet and then 300 µl of water was added and mixed thoroughly using a vortex mixer (model MX-5) to break up pellet.

To each tube at a time, 3.0 ml of scintillation fluid was added, then capped the test tube and was mixed using a vortex mixer (model MX-5) until the mixture had a uniform cloudy appearance. A test tube with the mixture was then placed in the analyzer (Charm II 6600 counter version 3.12) for 60 seconds to allow counting and read cpm on [3H] channel. In a case were the counted cpm was greater than and within 50 cpm of the control point then a recount was done (Science Inc, 2024d).

#### **4. 3.4.3.4 Control point set-up**

A total of 6 replicates of positive control standards were run in the analyzer and averaged the 6 cpm results. In an event any of the six determinations deviated by more than 23% from the calculated average, then that determination was replaced and then another averaging was done. After averaging, a 23% of the final average was added to calculate the control point (Science Inc, 2024d).

#### **4.3.4.4 Charm® II Aminoglycosides Test for Cow Milk at MRL**

##### **4.3.4.4.1 Zero Control Standard**

A zero-control standard supplied with the kit (certified organic whole milk powder) was used as negative control and was reconstituted with a 100 ml of 40°C water as indicated on label and then

was nicely shaken until all clumps were broken up. The remaining dry zero control standard was placed in the refrigerator while the reconstituted zero control standard was held in the refrigerator for a period of 72 hours (Science Inc, 2024a).

#### **4.3.4.4.2 Positive Control Standard**

A multi-antimicrobial standard (containing 30 ppb gentamicin when reconstituted) which was supplied with the kit (manufactured from USP reference standards) was reconstituted with a 100 ml of negative control or zero control standard. This mixture was nicely shaken and then allowed to stand in the refrigerator for 15 minutes before use. The remaining dry multi-antimicrobial standard was kept in the refrigerator while the reconstituted positive control standard was held in the refrigerator for only a period of 48 hours (Science Inc, 2024a).

#### **4.3.4.4.3 Aminoglycoside Test Competitive Assay-Procedure**

About  $\frac{3}{4}$  of a test tube was filled with a milk sample or control and was thoroughly mixed for 10 seconds and then was centrifuged (Rotofix 32A) for 3 minutes at 3400 rpm. The sample was immediately removed from the centrifuge and was cooled to 4°C. A white tablet was placed into an empty test tube and a 300  $\mu$ l of water was added and allowed to mix using a vortex mixer (model MX-5) for 10 seconds to completely break up tablet. Thereafter, 5.0 ml of centrifuged sample or control was collected from below the fat layer while using a new pipet tip for each sample and mix.

To this mixture a yellow tablet (containing 1.9 kilobecquerels (kBq) of [3H]-gentamicin) was added and was immediately mixed using a vortex mixer or by swirling milk up and down 15 times for 15 seconds. Thereafter, the mixture was incubated (Inctronic 2) at 35 °C for 3 minutes and centrifuged (Rotofix 32A) for 3 minutes at 3400 rpm. The mixture was immediately removed from the centrifuge and poured off milk and any possible fat ring which was present was broken with a cotton swab and the test tube was kept inverted. After removal of the fat ring and the test tube was wiped dry with a cotton swab without disturbing the pellet.

Then a 300  $\mu$ l of water was then added and mixed thoroughly using a vortex mixer to break up the pellet completely while ensuring that it was suspended in water before adding scintillation fluid. A 3.0 ml of scintillation fluid was added, then capped the test tube and was mixed using a vortex mixer until the mixture had a uniform cloudy appearance. A test tube with the mixture was then placed into the analyzer (Charm II 6600 counter version 3.12) for 60 seconds to allow counting and read cpm on [3H] channel. In a case were the counted cpm was greater than and within 50 cpm of the control point then a recount was done (Science Inc, 2024a)

#### **4.3.4.4.4 Control point set-up**

A total of 5 replicates of positive control standards were run in the analyzer instead of 6 positive standards and then averaged the 5 cpm results which were obtained. In a case where one of the five determinations deviated by more than 20% from the calculated average, then that determination was replaced and then another averaging was done. Thereafter, 20% to the final average was added to calculate the control point (Science Inc, 2024a).

#### **4.3.4.5 Charm® II Macrolides Test for Cow Milk at MRL**

##### **4.3.4.5.1 Zero control standard**

A zero-control standard supplied with the kit (certified organic whole milk powder, when reconstituted, it is comparable to milk from cows certified to be antimicrobial drug free) was used as negative control standard. A zero-control standard was reconstituted with a 100 ml of 40°C water as indicated on label and was nicely shaken until all clumps were broken up and then placed in the refrigerator before use. The dry zero control standard that remained was placed in the refrigerator while the reconstituted zero control standard was held in the refrigerator for a period of 72 hours (Science Inc, 2024b).

##### **4.3.4.5.2 Positive control standard**

A multi-antimicrobial standard (containing 50 ppb erythromycin when reconstituted) was supplied with ETBL- kits and manufactured from USP reference standards. A positive control standard was reconstituted with a 50 ml of negative control standard and was nicely shaken for 10 seconds to allow mixing and then placed in the refrigerator for 15 minutes. This reconstituted positive control standard mixture was mixed every time before use. The remaining dry positive control standard was kept in the refrigerator while the reconstituted positive control standard was held in the refrigerator for a period of 48 hours (Science Inc, 2024b).

##### **4.3.4.5.3 Macrolide Test Sequential Assay-Procedure**

A white tablet was added to an empty test tube and a 300 µl of water was added and mixed using a vortex mixer (model MX-5) for 10 seconds to completely break up tablet. Thereafter, a 5 ml of sample or control was added while using a new pipet tip for each sample. The mixture was then placed on the vortex mixer for 10 seconds and was incubated (Inctronic 2) at 65°C for 2 minutes. A green tablet (containing less than 0.3 kilobecquerels (kBq) of [14C]-erythromycin) was added and immediately mixed using a vortex mixer for 10 seconds.

The mixture was then incubated (Inctronic 2) at 65°C for 2 minutes and was centrifuged (Rotofix 32A) for 3 minutes at 3400 rpm. The mixture was immediately removed from the centrifuge and milk was poured off and then a fat ring was removed and the test tube was wiped dry with cotton swabs without disturbing the pellet. A 300 µl of water was then added and mixed thoroughly using a vortex mixer to completely break up the pellet. Then 3.0 ml of scintillation fluid was added, capped the test tube and mixed using a vortex mixer until a uniform cloudy appearance. The test tube with a mixture was placed in the analyzer (Charm II 6600 counter version 3.12) for 60 seconds and read cpm on [14C] channel (Science Inc, 2024b).

#### **4.3.4.5.4 Control point set-up**

A total of 6 replicates of zero control standards were run in the analyzer and then averaged the 6 cpm results. In a case were one of the six determinations deviated by more than 15% from the calculated average, then that determination was replaced and another averaging was done. A 35% of the final average was subtracted to calculate the control point (Science Inc, 2024b).

#### **4.4 Data management and analysis methods**

After the completion of data collection (qualitative) from laboratory analysis, data was entered on Microsoft Excel work-sheet® 2010 for cleaning and management and then exported to Stata/SE 14.2 for windows (64-bit x 86x64) for data analysis as similarly described by (Mwansa *et al.*, 2023). Questionnaire survey data collected using KoboToolbox (v2024.2.4) was first downloaded in Microsoft Excel work-sheet® 2010 for easier cleaning and management. Thereafter, this data was exported into Stata/SE 14.2 windows (64-bit x 86x64) for analysis.

The outcomes concerning knowledge questions were reclassified as “correct” when the response is “Yes” and “incorrect” when the response was “No”. The aggregate score for all knowledge questions would range from 0 to 4 points for a given participant. However, the outcome of questions for the attitude questions, when the response was “Yes or No” depended on the correct answer for the question itself. The aggregate score for all attitude questions would range from 0 to 5 points for a given participant. The outcome of questions for the practice questions, when the response was “Yes or No” depended on the correct answer for the question itself.

The aggregate score for all practice questions would range from 0 to 10 points for each given respondent. During the process of overall scoring for knowledge, attitude and practice regarding antibiotic use and residues, a benchmark of 70% was used to determine either “good” or “poor” (Mudenda *et al.*, 2023). Participants overall knowledge score was categorized as good and poor/bad

if the score was  $\geq 3$  points <3 points, respectively. The respondent's overall attitude score was categorized as good and poor/negative if the score was  $\geq 4$  points and <4 points, respectively.

Participants overall practice score was categorized as good and poor/bad if the score was  $\geq 7$  points <7 points, respectively. To reach a conclusive judgment for scoring, the proportion of respondents scoring above the mean scores was calculated relative to the total number of respondents in the district. If this proportion exceeded 70%, the population was classified as having good KAP and if it fell below 70%, the population was considered to have poor KAP.

This study utilized the Pearson chi-square ( $X^2$ ) test to assess statistical significance concerning antibiotic residues between the Mbala and Kasama districts according to (Gonah *et al.*, 2024). Pearson's chi-square test was used to identify significant differences in KAP variables between the Mbala and Kasama districts. Pearson's chi-square ( $X^2$ ) test was also used to establish a significance difference in categorized knowledge, attitudes, and practices scores between the groups with good and poor outcome scores in Mbala and Kasama.

Additionally, Pearson correlation (r) test was used to establish relationships between total scores of knowledge-attitude, knowledge-practice and attitude-practice (Mudenda *et al.*, 2022). The study utilised multivariable linear regression to establish significant relationships between knowledge, attitude and practice scores with sociodemographic data (Mudenda, *et al.*, 2022). Descriptive analysis was also employed to summarise the collected data and express it in terms of tables, charts and percentages (%) of each variable of interest. During analysis, a  $p=0.05$  (Confidence Interval CI 95%) was used as cut-off point for significance difference as described by (Mohamed *et al.*, 2020).

#### **4. 5 Expected results**

Upon completion of this research, it was anticipated to thoroughly analyse all collected raw cow milk samples, enabling identification of various classes of antibiotic residues. Not only did this study detect the presence of antibiotic classes residues but also measured the level of knowledge, attitude and practice regarding antibiotic use and residues among smallholder dairy producers. It was also expected to determine the prevalence of antibiotic class residues in milk samples. The study was also expected to identify gaps in knowledge and compliance regarding antibiotic use and withdrawal periods.

Additionally, this study provided scientific-evidence to make recommendations for improved policy and regulations and “One Health” strategies that promote antibiotic stewardship among smallholder dairy producers through farmer education or training programs, regulatory controls, market developments and surveillance and monitoring of antibiotic residues in milk to ensure food safety.

## **5. ETHICAL CONSIDERATION**

### **5.1 Ethical approval**

Authority to conduct research in Zambia was obtained from the ethics committee under the Directorate of veterinary services, Ministry of Fisheries and Livestock, Mulungushi house, Lusaka, Zambia.

### **5.2 Participants concern and applied ethics**

Before collection of milk samples or questionnaire survey, an informed verbal statement of consent was obtained from study participants. In addition, the study made sure that rights of respondents were protected and all target farmers were free to accept or decline to participate in the study. No incentives were provided for consenting to participate in this study. To allow confidentiality, all the study participants were treated as anonymous agents and were allocated with study specific ID number.

## **6. RESULTS**

This section captures the entire results of laboratory analysis starting from setting up of the critical controls points using the Charm II analyser and analysis of each milk sample for every class of antibiotic namely; beta-lactams, tetracyclines, macrolides, sulphonamides and aminoglycosides. This section also incorporates results concerning a questionnaire survey and all applied statistical analysis for both laboratory and questionnaire survey data.

### **6.1 Critical control points set-up for antibiotic classes residues**

Results on setting-up critical control points are presented in Table 4, where a total of 6 positive standards control replicates were utilized for tetracycline, beta-lactams, and sulphonamides. In contrast, macrolides employed 6 negative control standard replicates. Additionally, aminoglycosides were modified to use 5 positive standard control replicates instead of 6 Table 4. Although analysis of all the five classes of antibiotics was intended to use three (3) comparative standards during validation, this study only utilized one (1) control standard for each class of antibiotic.

Table 4: Establishment of Critical Control Points for antibiotic class residues (CCP)

<b>Class of antibiotic</b>	<b>Number of standards used</b>	<b>Type of control used</b>	<b>Standard counts per minutes (cpm)</b>	<b>cpm selected for averaging</b>	<b>Average cpm</b>	<b>Inclusion or exclusion (%) to average cpm</b>	<b>Critical Control Point</b>	<b>Zero control (cpm)</b>	<b>Positive control (cpm)</b>
Tetracycline	6	Positive	918; 1019; 965; 1028; 907; 990	918; 1019; 965; 1028; 907; 990	971	25% addition	<b>1194</b>	2065	
Macrolides	6	Negative	1200; 1253; 1257; 1321; 2121; 1245	1200; 1253; 1257; 1321; 1245	1255	35% subtraction	<b>815</b>		724
Beta-lactams	6	Positive	418; 429; 456; 279; 193; 151	418; 429; 456	434	15% addition	<b>499</b>	1501	
Sulphonamides	6	Positive	490; 585; 545; 547; 603; 555	585; 545; 547; 555	558	24% addition	<b>692</b>	1150	
Aminoglycosides	5	Positive	521; 514; 583; 609; 589	521; 514; 583; 609; 589	563	20% addition	<b>676</b>	1386	

## 6.2 Prevalence of antibiotic class residues in raw cow milk

Results on assessment of five (5) antibiotic class residues in milk samples collected from Mbala and Kasama district are presented in Table 5. Overall, a total of 85 milk samples (91.4%) tested positive for at least one class of antibiotic residues. The prevalence in Mbala was 98.1% (n = 53) and Kasama was 82.0% (n = 32). The proportion of milk samples containing antibiotic residues differed significantly between the two districts ( $\chi^2 = 7.4630$ ,  $p=0.006$ ). Among the residues detected, sulphonamides were the most prevalent at 68.8% (n = 64), followed by macrolides at 58.1% (n = 54), tetracyclines at 12.9% (n = 12), beta-lactams at 9.7% (n = 9), and aminoglycosides at 2.2% (n = 2), Table 5.

In Mbala, 87.0% (n = 47) of the milk samples contained sulphonamides, compared to 43.6% (n = 17) in Kasama. The difference in the number of samples positive for sulphonamides between the two districts was significant ( $\chi^2 = 19.9200$ ,  $p<0.001$ ). Macrolides were detected in 59.3% (n = 32) of milk samples in Mbala and 56.4% (n = 22) in Kasama tested positive, with no significant difference observed between the districts ( $\chi^2 = 0.784$ ,  $p=0.0755$ ). The prevalence of tetracycline residues was 7.4% in Mbala and 20.5% in Kasama, showing no significant difference ( $\chi^2 = 3.4608$ ,  $p=0.063$ ), Table 5.

The proportion of milk samples containing beta-lactams was 11.1% (n = 6) in Mbala and 7.7% (n = 3) in Kasama, revealing no significant difference between the two districts ( $\chi^2 = 2.3968$ ,  $p=0.122$ ). Lastly, the presence of aminoglycosides was found in 3.7% (n = 2) of samples from Mbala, while no samples from Kasama tested positive, with no significant difference noted ( $\chi^2 = 1.4762$ ,  $p=0.224$ ), Table 5.

Table 5: Prevalence of antibiotic class residues %(n) in milk samples collected from Mbala and Kasama districts.

District	% (n)	% (n)	% (n) Positives antibiotic classes				
			Tetracycline	Macrolides	Beta-lactams	Sulphonamides	Aminoglycosides
	Overall Tested samples	Overall positive samples					
Mbala	58.1(54)	98.1 (53)	7.4(4)	59.3(32)	11.1(6)	87.0(47)	3.7(2)
Kasama	41.9(39)	82.0 (32)	20.5(8)	56.4(22)	7.7(3)	43.6(17)	0 (0)
<b>Total</b>	100(93)	91.4 (85)	12.9(12)	58.1(54)	9.7(9)	68.8(64)	2.2(2)
<b>P-Value</b>		<b>0.006*</b>	0.063	0.784	0.122	<b>0.000*</b>	0.224
<b>Pearson chi2 (<math>\chi^2</math>)</b>		7.4630	3.4608	0.0755	2.3968	19.9200	1.4762

Significant *p* -Value < 0.05; Pearson chi2 test ( $\chi^2$ )

### 6.3 Socio-demographic and economic characteristics of smallholder farmers during survey

The socio-demographic characteristics of respondents interviewed in Mbala and Kasama during the questionnaire survey are presented in Table 6. A total of 100% (n=101) smallholder dairy farmers (respondents) produced milk at small-scale in both Mbala 41.6% (n=42) and Kasama 58.4% (n=59) participated in the questionnaire survey Table 6. Among them, 74.3% (n=75) were male and 25.7% (n=26) were female. According to districts Mbala had 88.1% (n=37) males and 11.9% (n=5) females while for Kasama their where 64.4% (n=38) males and 35.6% (n=21) were females.

Their ages ranged from 27 to 86 years, with a mean age of 50.6 years and a standard deviation of 12.58, at a 95% Confidence level of 2.48. The majority had attended secondary education 42.6% (n = 43), followed by primary 41% (n = 40), tertiary 9.9% (n = 10), and no formal education 6.9% (n = 7). Most of these farmers were engaged in gainful employment particularly farming 63.4% (n = 64), while the remaining 36.6% (n = 37) were not Table 6. In terms of dairy farming experience, the majority had between 1 to 5 years of experience 69.3% (n = 69), followed by 5 to 10 years 15.8% (n = 16), over 10 years 13.9% (n = 14), and less than 1 year 2.0% (n= 2), as illustrated in Table 6.

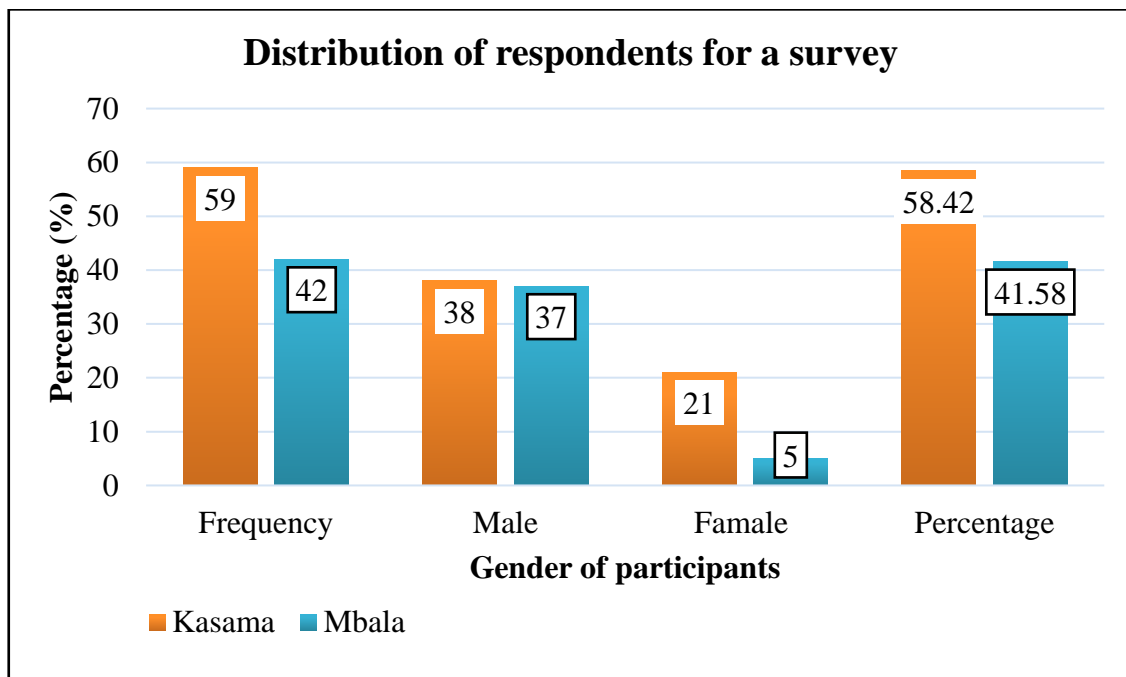


Figure 4. Distribution of respondent's gender during questionnaire survey

Table 6: Socio-demographic and economic characteristics of the participants interviewed during a questionnaire survey.

<b>Characteristics</b>	<b>Frequency (n=101)</b>	<b>Percentage (%)</b>
<b>Sex</b>		
Male	75	74.3
Female	26	25.7
<b>Age</b>		
18-34 years	14	13.9
35-44 years	14	13.9
45 years and above	73	72.3
<b>Level of education</b>		
None	7	6.9
Primary school	40	41
Secondary school	43	42.6
Tertiary	10	9.9
<b>Gainful employment</b>		
Yes	64	63.4
No	37	36.6
<b>Experience in dairy farming</b>		
Less than 1 year	2	2.0
Above 1-5 years	69	69.3
Above 5-10 years	16	15.8
Above 10 years	14	13.9
<b>Reason for Dairy farming</b>		
Consumption	6	5.9
Sale	0	0
Consumption and sale	95	94.1
<b>Monthly income</b>		
Less than K500	16	15.8
Above K500-1000	43	42.6
Above K1000-5000	32	31.7
Above K5000	10	9.9

#### 6.4 Assessing the levels of knowledge, attitude and practice about antibiotic use and residues

Table 7: The proportion of dairy producers who answered correctly and incorrectly according to knowledge, attitude and practice questions.

Awareness about antibiotic use and residues	Responses for Kasama		Responses for Mbala		Overall response		Overall percentage		X <sup>2</sup>	P-Value
	Yes	No	Yes	No	Yes	No	%	%		
<b>Knowledge</b>										
Do you know what antibiotics are and their use?	28	31	36	6	64	37	63.37	36.63	15.469	0.000*
Do you know what are antibiotic residues	19	40	3	39	22	79	21.78	78.22	9.044	0.003*
Do you know what is antibiotic withdraw period?	38	21	23	19	61	40	60.40	39.60	0.954	0.329
Do you know how to determine the withdrawal period for an antibiotic used in animal treatment?	28	31	22	20	50	51	49.50	50.50	0.238	0.626
<b>Attitude</b>										
Can giving of antibiotics to animals that are not sick make them grow faster and produce more milk?	21	38	7	35	28	73	27.72	72.28	4.3862	0.036*
Should a farmer observe a withdrawal period for antibiotics in a milking animal?	58	1	36	6	94	7	93.07	6.93	6.0298	0.014*
Can antibiotic residues be present in milk for consumers if the animals producing that milk are being treated with antibiotics or did not adhere to the withdrawal period?	49	10	16	26	65	36	64.36	35.64	21.6160	0.000*

Can continuous consumption of antibiotic residues in animal source foods above MRLs cause side effects to humans?	53	6	19	23	72	29	71.29	28.71	23.8349	0.000*
Should a farmer get a prescription from a veterinarian before purchasing an antibiotic?	58	1	42	0	100	1	99.01	0.99	0.7190	0.396
<b>Practice</b>										
Do you always use antibiotics for any kind of disease treatment?	40	19	40	2	80	21	79.21	20.79	11.2183	0.001*
Do you use antibiotics as a prophylactic measure?	38	21	32	10	70	31	69.31	30.69	1.6015	0.206
Do you use antibiotics to increase your milk production?	13	46	5	37	18	83	17.82	82.18	1.7188	0.190
Do you observe withdraw period when your dairy cattle are on treatment?	52	7	27	15	79	22	78.22	21.78	8.1912	0.004*
Do you consult with your area Veterinary assistant for a prescription before obtaining antibiotics?	58	1	36	6	94	7	93.07	6.93	6.0298	0.014*
Do you treat animals on your own when they are sick?	11	48	21	21	32	69	31.68	68.32	11.1446	0.001*
Do you engage your area veterinary assistant to treat your sick animals?	57	2	37	5	94	7	93.07	6.93	2.7578	0.097
Do you participate in livestock disease control trainings?	52	7	34	8	86	15	85.15	14.85	1.0011	0.317
Do you keep records of treatment for animals at your farm?	16	43	5	37	21	80	20.79	79.21	3.4482	0.063

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Do you screen for antibiotic residues in the milk you produce?	0	59	0	42	0	101	0	100
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Significant  $p$  -Value < 0.05;  $X^2$  = Chi square test

#### **6.4.1 Knowledge regarding antibiotic use and residues**

Around 47.5% and 85.7% of respondents in Kasama and Mbala respectively reported to know what antibiotics and their use with a significance difference ( $p < 0.001$ ). About 32.3% of respondents in Kasama and 7.1% Mbala district reported about knowing what are antibiotic residues with a significance difference ( $p = 0.003$ ). Overall, proportion of 60.4% of respondents from both districts reported to know what is antibiotic withdraw period, with no significance difference ( $p = 0.329$ ). Only a proportion of 49.5% of smallholder dairy producers from both districts agreed to know how to determine antibiotic withdraw period, with no significance difference ( $p = 0.626$ ), Table 7.

#### **6.4.2 Attitudes regarding antibiotic use and residues**

A proportion of 35.6% and 16.7% participants in Kasama and Mbala respectively had a perception that giving of antibiotics to animals that are not sick make them grow faster and produce more milk. There was a proportional difference between two districts ( $p = 0.036$ ). A total of 98.3% of respondents in Kasama district and 85.7% of respondents in Mbala district had a perception that a farmer should observe a withdrawal period for antibiotics in a milking animal since the milk might be contaminated. There was a notable or significance difference between the two districts ( $p = 0.014$ ), Table 7.

Around 83.1% of participants in Kasama and 38.1% of Mbala agreed that antibiotic residues can be present in milk for consumers if the animals producing that milk are being treated with antibiotics or did not adhere to the withdrawal period. There was a significance difference between the two districts ( $p < 0.001$ ) Table 7. About 89.8% and 45.2% of respondents from Kasama and Mbala district respectively had a perception that continuous consumption of antibiotic residues in ASF above MRLs could cause side effects to humans due to residues present. There was a notable difference between the two districts ( $p < 0.001$ ) Table 7.

#### **6.4.3 Practices regarding antibiotic use and residues**

Around 67.8% of respondents in Kasama and 95.2% from Mbala agreed of always using antibiotics for any kind of disease treatment, with a significance difference ( $p = 0.001$ ). About 88.1% and 64.3% of respondents from Kasama and Mbala district respectively reported to observe withdraw period when their dairy cattle are on treatment, with proportional significance difference ( $p = 0.004$ ). A proportion of 98.3% of participants from Kasama and 85.7% from Mbala agreed to that they consult with their area Veterinary assistant for a prescription before obtaining antibiotics Table 7. There was a notable proportional statistical difference ( $p = 0.014$ ) between both districts.

About 18.6% of respondents from Kasama and 50% from Mbala district agreed treating sick animals (dairy cattle) on their own. There was a reported proportional significance difference ( $p=0.001$ ) between the two districts. An overall of 79.2% of respondents from both districts agreed not keeping records of treatment for their animals and 100% of respondents did not screen for antibiotic residues in the milk being produced Table 7.

### 6.5 The correlation between KAP regarding antibiotic use and residues

Kasama district showed no any correlation and significance relationship among knowledge-attitude ( $r=0.255$ ,  $p=0.052$ ), knowledge-practice ( $r=0.195$ ,  $p=0.138$ ), and attitude-practices ( $r=0.151$ ,  $p=0.254$ ) while Mbala district reported a strong correlation and significance relationship among knowledge-attitude ( $r=0.687$ ,  $p<0.001$ ), knowledge-practice ( $r=0.470$ ,  $p=0.002$ ) and attitude-practice ( $r=0.629$ ,  $p<0.001$ ), Table 8.

Table 8: Pearson correlation and significance levels for knowledge, attitude and practice scores between Kasama and Mbala district.

KAP scores	Kasama district		Mbala district	
	<b>Correlation coefficient</b>	<b>p-Value</b>	<b>Correlation coefficient</b>	<b>p-Value</b>
Knowledge-attitude	<b>0.255</b>	<b>0.052</b>	<b>0.687</b>	<b>0.000*</b>
Knowledge-practice	<b>0.195</b>	<b>0.138</b>	<b>0.470</b>	<b>0.002*</b>
Attitude-practice	<b>0.151</b>	<b>0.254</b>	<b>0.629</b>	<b>0.000*</b>

### 6.6 Overall KAP scores about antibiotic use and residues.

Table 9: Overall KAP scores for smallholder dairy producers regarding antibiotic use and residues in Mbala and Kasama district.

Variable	Kasama district (n=59)			Mbala district (n=42)		
	<b>Target %(n)</b>	<b>Good %(n)</b>	<b>Poor</b>	<b>Target</b>	<b>Good</b>	<b>Poor</b>
<b>Knowledge</b>	70(41)	52.5(31)	47.5% (28)	70(29)	61.9(26)	38.1(16)
<b>Attitude</b>	70(41)	57.6(34)	42.4(25)	70(29)	47.6(20)	52.4(22)
<b>Practice</b>	70(41)	40(23)	60(36)	70(29)	35.7(15)	64.3(27)

In this study only 52.5% of respondents in Kasama reported to have good knowledge while 47.5% had poor knowledge regarding antibiotic use and residues. About 57.6% the same respondents of Kasama had good/positive attitude scores while the remaining 42.4% scored with poor/negative attitude regarding antibiotic use and residues. Around 40% of respondents from Kasama reported with good practices towards antibiotic use and residues while the other 60% had poor practices regarding antibiotic use and residues. In comparing 61.6% of respondents in Mbala district scored for good knowledge and 38.1% had poor/bad knowledge about antibiotic use and residues Table 9.

Additionally, about 47.6% of respondents of Mbala district had good/positive attitude towards antibiotic use and residues while the other 52.4% displayed with poor/negative attitude. Around 35.7% of respondents in Mbala districts had good practices regarding antibiotic use and residues while the rest of 64.3% had poor/bad practices Table 9. These findings indicated that overall, smallholder dairy producers had poor knowledge, negative attitude and poor practices since the KAP score were below the expected 70% frequency.

Table 10: Linear regression of socio-demographic characteristics and knowledge, attitude, and practice scores of the smallholder dairy producers.

<b>Sociodemographic characteristics</b>	<b>Kasama district</b>			<b>Mbala district</b>		
	Knowledge	Attitude	Practice	Knowledge	Attitude	Practice
<b>Age</b>	0.013*	0.298	0.191	0.005*	0.100	0.818
<b>Sex</b>	0.016*	0.386	0.920	0.163	0.098	0.722
<b>Educational level</b>	0.001*	0.287	0.378	0.000*	0.006*	0.027*
<b>Experience in dairy farming</b>	0.441	0.674	0.297	0.113	0.709	0.594

This study utilized a multivariable linear regression to assess significant relations or influences between socio-demographic characteristics of respondents and their knowledge, attitude and practice scores. The age of respondents in both Kasama and Mbala district reported to have a statistically significant influence ( $p=0.013$ ) and ( $p=0.005$ ) respectively on the knowledge regarding antibiotic use and residues in dairy production. On the other hand, the sex or gender of respondent's in Kasama district appeared to significantly influence ( $p=0.016$ ) their knowledge Table 10.

In addition, the educational levels of respondent's in Kasama appeared to significantly influence ( $p=0.001$ ) their knowledge towards antibiotic use and residues. The level of educational among farmers in Mbala district appeared to significantly influenced all their knowledge ( $p<0.001$ ), attitude ( $p=0.006$ ) and practices ( $p=0.027$ ). These findings implied that lower education levels among smallholder dairy producers was associated with poor KAP towards antibiotic use and residues Table 10.

Additionally, after checking for relationships between socio-demographic data with categorized KAP scores using multivariable linear regression as shown in Table 10 for both districts, all categorical variables that showed a significant relationship were explored further using Pearson chi2 in order to determine which specific category within the socio-demographic data that had influence on those particular KAP scores (Table 11).

Table 11: Relationship between significant socio-demographic characteristics with good and poor knowledge, attitude and practices scores.

Socio-demographic data	Knowledge scores Kasama			Knowledge scores Mbala			Attitude scores Mbala			Practice scores Mbala		
	Good	Poor	P-Value	Good	Poor	P-Value	Good	Poor	P-Value	Good	Poor	P-Value
<b>Age</b>			0.028*			0.054						
Below 30 years	0	2		1	1		-	-		-	-	-
Above 30 to 50 years	10	16		7	10		-	-		-	-	-
Above 50 years	21	10		18	5		-	-		-	-	-
<b>Sex</b>			0.006*									
Male	25	13		-	-		-	-		-	-	
Female	6	15		-	-		-	-		-	-	
<b>Education level</b>			0.000*			0.004*			0.434			0.096
None	1	5		0	1		0	1		0	1	
Primary	5	17		7	12		8	11		4	15	
Secondary	23	5		12	3		7	8		6	9	
Tertiary	2	1		7	0		5	2		5	2	

Significant  $p$  -Value < 0.05;  $X^2$  = Chi square test

Respondents from Kasama showed their age, sex and educational had a significant influence on their knowledge. Those aged especially 30 to 50 years ( $p=0.028$ ), sex (majority of females) ( $p=0.006$ ) and low education levels (none to primary) ( $p<0.001$ ) were significantly linked with poor knowledge regarding antibiotic use and residues. The majority of respondents among those with poor knowledge (62.5%) in Mbala district were aged between 30 to 50 years. Those with poor knowledge (75%) were also associated ( $p=0.004$ ) with low education (none to primary). Majority of those with negative attitude (50%) and poor practices (55.6%) in Mbala were associated with none to primary education levels (Table 11).

Table 12: Overall KAP about antibiotic use and residues in Mbala and Kasama.

Scores	Mbala district	Kasama district	Chi2	P=Value
<b>Knowledge</b>			0.8747	0.350
Good	26	31		
Poor	16	28		
<b>Attitude</b>			0.9877	0.320
Good	20	34		
Poor	22	25		
<b>Practice</b>			0.1117	0.738
Good	15	23		
Poor	27	36		

Significant  $p$  -Value < 0.05;  $X^2$  = Chi square test

Overall, there was no any significance difference in terms of knowledge ( $p=0.350$ ), attitude ( $p=0.320$ ) and practices ( $p=0.738$ ) between respondents of Kasama and Mbala district. This implied that smallholder dairy producers from both districts had similar KAP towards antibiotic use and residues Table 12.

### 6.7 Commonly used antibiotics reported during questionnaire survey

The results for antibiotics reported to be commonly used by smallholder producers are shown in Figure 5. Most respondents during this survey indicated that oxytetracycline (45%) and sulphonamides (19%), aminoglycosides (9%), tyrosine (macrolides) 8%, and amoxicillin (beta-lactams) 7% were among the most frequently utilized for disease treatment.

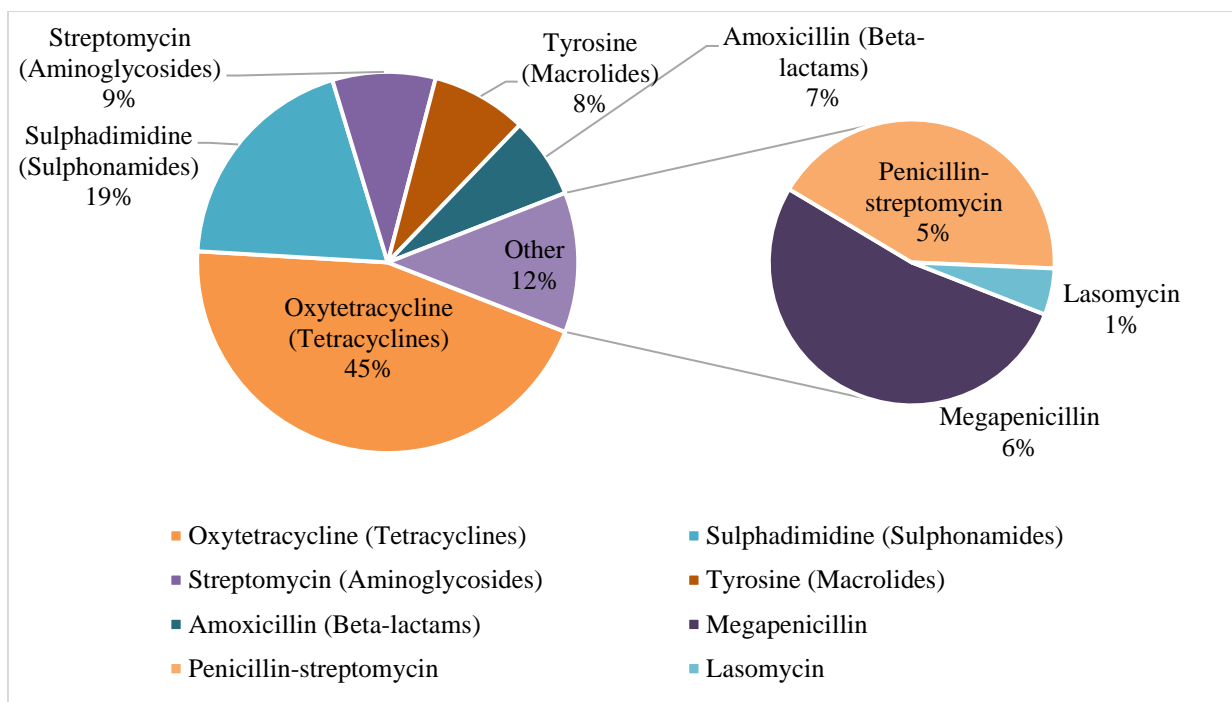


Figure 5. Commonly used antibiotics among farmers in both Mbala and Kasama

### 6.8 Sources of used antibiotics among smallholder dairy producers in Mbala and Kasama district

The chart in below illustrates the sources of antibiotics used, expressed in percentages. The agro-vet shops emerged as the primary source, accounting for 47.5% of the total, followed by veterinary or livestock officials at 34.6%. Sourcing from other farmers contributed 10.5% as a source, while pharmacies represented the least at 7.4% Figure 6.

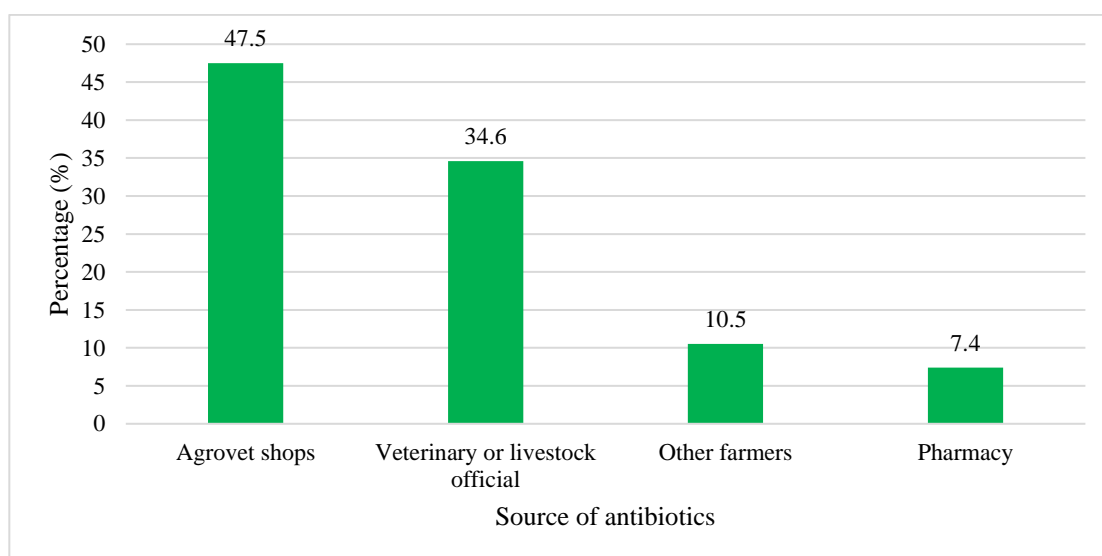


Figure 6. Common sources of antibiotics amongst smallholder dairy farmer

## 7. DISCUSSION

To the best of our knowledge, this study presents the first-ever report of antibiotic class residues in raw cow milk sampled from individual cattle and assess KAP regarding antibiotic use and antibiotic residues among smallholder dairy producers in Northern province, Zambia. In this study five (5) main antibiotic class residues including, tetracyclines, beta-lactams, sulphonamides, macrolides and aminoglycosides were assessed in milk samples from both Kasama and Mbala district, Northern, Zambia. This study also managed to evaluate KAP about antibiotic use and residues were conducted among smallholder dairy producers.

Milk is considered one of the most consumed nutritious food products in the market containing essential and an excellent source of many minerals, vitamins, proteins (amino acids) and sugar and along with dairy products remains essential for the human diet (Pereira, 2014). However, antibiotic residues are commonly deposited in ASF such as milk, meat, eggs, poultry and fish and this is precipitated by inappropriate dispensing, non-compliance to recommended antibiotic withdraw period, poor regulations and misuse or overuse of antibiotics in animals during treatments, prophylaxis and growth promotion (Mainda, 2016; Mudenda, Malama, *et al.*, 2022).

In this study a Charm II competitive receptor assay was used to detect antibiotic class residues in milk samples. Charm II test is a rapid immunoreceptor assay which detects several antibiotic class residues at EU/MRL in raw commingled and pasteurized cow milk and designed for use by dairy, veterinary, laboratory, field, and regulatory personnel (Science Inc, 2011). Smallholder dairy producers in the regions of Mbala and Kasama mostly produce milk on a small scale, with an average of one (1) milking cow per farmer. This explains why samples were chosen to be taken from individual animals because milk is rarely sold as bulk because there are so few animals that produce milk at each farmstead. This study focussed on analysis of only milk samples collected from individual animals because bulking milk can reduce the levels of antibiotic residues to undetectable levels, as also reported by (Ouma *et al.*, 2021).

This study revealed that majority of milk samples were tested positive for at least one class of antibiotic residues. Notably, milk samples from Mbala showed a higher prevalence of antibiotic residues compared to Kasama with a notable significance difference Table 5. This discrepancy may be attributed to farmers in Kasama being somewhat more compliant with the antibiotic withdrawal period than those in Mbala. Among all antibiotic classes, only sulphonamides among all detected antibiotic classes reported with significance difference between districts. This means that

sulphonamides were mostly used among smallholder dairy producers from Mbala than those of Kasama.

Overall, the prevalence of antibiotic residues above EU/MRLs Table 5 in milk from both districts was extremely concerning and a possible driver of AMR and other public health threats to consumers in Kasama and Mbala district. The presence of antibiotic residues in milk samples was more in sample from Mbala than Kasama. This suggests poor knowledge, negative attitude and poor practices among smallholder dairy producers. These producers displayed non-compliance with antibiotic withdrawal period, poor regulatory controls, lack of surveillance of antibiotic residues and lack of awareness in the use of antibiotics is more in Mbala than Kasama.

In addition to the above, classes of antibiotic residues like sulphonamides and macrolides were the most prevalent antibiotic residues in majority of milk samples. Therefore, this was a clear indication of their frequent usage among smallholder dairy producers in both Kasama and Mbala. This could also report their misuse and overuse during dairy production. The other antibiotic class residues detected in milk samples included; tetracyclines, beta-lactams and aminoglycosides.

Following the detection of five (5) different classes of antibiotic residues in milk samples, the results were compared to those reported by other researchers. Sulphonamides based drugs are amongst the common used antibiotic by cattle farmers in Zambia which aligned with this study laboratory findings (Mainda *et al.*, 2015). A high prevalence of macrolides in this study could be an indication of its common use and an alternative drug of choice among smallholder dairy producers within both districts. This finding is consistent with a study conducted in Guelma, Algeria, by Layada *et al.* (2016), which identified macrolides as the second most prevalent in cow milk, following  $\beta$ -lactams.

Despite, the fact that tetracycline and beta-lactams indicated a low prevalence, they are still considered as commonly used antibiotics in Zambia among livestock farmers (Mainda *et al.*, 2015). This study finding could perhaps mean a reduction in the use of the common drugs among smallholder dairy producers during the time of sample collection. A study conducted in Zimbabwe by Chabanga (2024) showed majority presence of penicillin's (beta-lactams) with 44.9% and tetracyclines 34.7% whereas sulphonamides and macrolides were most prevalent in this study, implying a shift from the use of common tetracyclines and penicillin's to other types of alternative antibiotics.

Additionally, this could imply a possibility of farmers unable to establish a difference among antibiotic during use. The prevalence of antibiotic residues in this study was higher than a similar recent study conducted in Lusaka province, Zambia by Kunda (2015) which indicated 30.1% of positive bulk milk samples collected among smallholder dairy producers. In comparison to present this study, it could imply an increase in the misuse and overuse of antibiotics among smallholder dairy producers in Zambia since the prevalence was higher than a previous related study.

The contamination of raw milk samples in this study was also higher than the 4.5% prevalence reported in Tanzania by Mdegela *et al.* (2009), 24% reported in Somalia by Mohamed *et al.* (2020), 7% reported in Tanzania by Kivaria *et al.* (2006) and a prevalence of 10.8% in Kenya above MRLs (Ouma *et al.*, 2021). The findings of this study were also greater than those of a previous study conducted in Himachal Pradesh, India, by Kumar *et al.* (2022) which reported 1.7% Oxytetracycline and 1.2% amoxicillin in milk samples, both exceeding the permissible limits. This implies that there was high misuse and overuse of antibiotics among smallholder dairy producers.

Additionally, this is coupled with low awareness, poor KAP about antibiotic use, residues and AMR in milk and other ASF in Kasama and Mbala. A study conducted in Brazil by Valença *et al.* (2021) reported 0.5% above MRLs an indication of low rate of violation of the established regulations of antibiotic residues which was not close with findings in this study. This means that in this study there was a higher violation of established regulations to ensure food safety. The results in this study were slightly higher than in Algeria by Layada *et al.* (2016) which reported prevalence of 65.5% in milk with penicillin, tetracyclines, macrolides, sulphonamides and quinolones above MRLs.

The higher prevalence of antibiotic residues in this study implies evidence of non-adherence to antibiotic withdraw period during dairy production. A study in Bangladesh by Chowdhury *et al.* (2015) revealed 24% beta-lactams and 16.5% tetracyclines in milk samples which were slightly higher than findings in this study. This reviewed a low use of tetracyclines and penicillins among employed smallholder dairy producers especially during season of sample collection. Majority of respondent's during a survey reported to mostly use these drugs for treatment during rainy season following an increase in disease burden.

A study conducted in Kenya indicated tetracyclines to be the most prevalent (33%) in milk samples than beta-lactams (10.5%) and sulphonamides (2%) above MRLs Odeny *et al.* (2024), contradicted with this study finding where sulphonamides were most prevalent and tetracyclines had low

prevalence Table 5. The prevalence of beta-lactams in this study was similar to a study in Kenya (Odeny *et al.*, 2024). This perhaps means a higher use of sulphonamides than beta-lactams and tetracyclines in disease management during the study period despite beta-lactams being widely used in disease treatment like mastitis (Ndungu *et al.*, 2021).

The overall prevalence of antibiotic residues in this study Table 5 was close to a study conducted by Gwandu *et al.* (2018) in Tanzania which reported a prevalence of 83%. This alarming prevalence of antibiotic residues in both studies could be attributed to poor regulation, improper monitoring system by smallholder dairy producers to follow prescribed drug withdraw period, lack of awareness on antibiotic residues and their impact, indiscriminate use (misuse and overuse) and lack of regular surveillance and monitoring practices.

To add on, this research aligns with a study in Enugu State, Nigeria, which also highlighted a prevalent unethical use of antimicrobial drugs among dairy producers (Njoga *et al.*, 2018). The presence of high levels of antibiotic residue in milk constitutes a serious public health hazard. In both districts no antibiotic residue screening tests were being used, thereby exposing consumers to the risk of ARs in milk and other ASF. Production of raw milk and related ASF with acceptable level of ARs below MRLs is essential to assure food safety and can be achieved through required procedures and antimicrobial drug use at farm level should be adopted as overall strategy (Codex, 2001).

Studies have shown that the presence of antibiotic residues in ASF above the MRL has been recognized worldwide by various public authorities due to public health threats like development of AMR to humans and animals (Kempe *et al.*, 2000). Therefore, emphasizing a requirement for rigorous and dependable methods for detecting antibiotic residues in milk and its products (Treiber & Beranek-Knauer, 2021). However, this study found that majority of milk samples contained antibiotic residues at or above the recommended EU/MRLs, which contradicts these established standards.

In addition, Zambia has a standardization body called the Zambia Bureau of Standards (ZABS) which focuses on standardization, food safety, standards development, quality control, quality assurance, and performing quality inspections for both imports and exports. Zambia also has the Food Safety Act No. 7 of 2019 National Assembly of Zambia, (2019), which prohibits the sale of any food that does not meet safety and quality requirements to protect public health. Despite these regulations, regulatory control in the use of antimicrobials still remains poor in Zambia which might significantly contribute to presence of forbidden ARs levels in ASF (Mainda *et al.*, 2015).

Recent studies have shown that Zambia, has limited information on antibiotic use and residues, poor regulation and monitoring and surveillance protocols system of antibiotics (Mainda *et al.*, 2015). Other recent studies conducted by Chowdhury *et al.* (2015) show that antibiotic residues in ASF especially milk, meat and egg is an important problem in for most developing countries. The public health implications of ARs deposition in ASF above MRLs include; toxicity of bone marrow, destruction of microflora in human intestinal microflora, allergic reactions, cancer and development and sustenance AMR (antibiotic-resistant bacteria and antibiotic resistance genes) (Cinquina *et al.*, 2003).

Additionally, the emergence and spread of AMR organisms, particularly those resistant to crucial medicinal antimicrobials, pose significant clinical challenges in both human and veterinary medicine. These issues are linked to elevated morbidity and mortality rates, a higher likelihood of treatment failure, and increased treatment costs associated with these organisms (Njoga *et al.*, 2018). The economic burden caused by infections with antibiotic resistant bacteria is estimated in the EU at EUR 1.5 billion per year in healthcare costs and productivity losses and \$55 billion in the U.S (Mobarki *et al.*, 2019; WHO, 2019).

This study also incorporated a questionnaire survey which aimed to assess KAP regarding antibiotic use and residues in milk among smallholder dairy producers within Mbala and Kasama district. The knowledge, attitudes, and practices of livestock farmers concerning antibiotic use and residues in animals are crucial factors in the emergence of AMR. The presence of antibiotic residues in ASF is amongst the leading cause of AMR which is among the top ten (10) global public health threat (WHO, 2019).

A higher proportion of respondents who participated in the questionnaire survey were from Kasama than Mbala district. Overall, most of these respondents were male, aged above 45 years, with none to primary education level, gainful employments through farming and had been in dairy farming for 1-5 years Table 6. Majority of the participants reported that the milk produced was meant for their personal consumption and at times sale. The implied a risk of AMR among consumers and direct toxicity since majority of milk samples tested were positive for antibiotic residues.

Overall, this study revealed that smallholder dairy producers in both districts exhibited poor knowledge, negative attitudes, and poor practices regarding antibiotic use and residues in their dairy

production. This was indicated by KAP scores falling below the expected threshold of 70% frequency Table 9. The findings also showed that the KAP levels among smallholder dairy producers from Kasama and Mbala district were not significantly different Table 12. This suggested that these participants had comparable levels of KAP, which matched with the concerning presence of antibiotic class residues in milk samples.

In addition, the overall KAP scores for both Kasama and Mbala district the target meant that smallholder dairy producers had a high likelihood of antibiotic misuse and overuse which could subsequently lead to antibiotic deposition in the milk being produced. The higher prevalence of antibiotic residues in milk samples from both districts correlated with their KAP scores. The weak correlation among KAP scores of smallholder dairy producers of Kasama meant that their level of knowledge had no influence on their attitudes and practices while attitude had no any influence on their practices Table 8.

In contrast, the correlation among KAP scores of smallholder dairy producers of Mbala were strong and significant meanings that their knowledge had an influence their attitudes and practices while attitudes also had an influence on their practices Table 8. Respondents in Kasama showed that their age, sex and educational level had a significant influence on their knowledge Table 10. Those aged 30-50 years, sex (majority females) and low education levels (none to primary) were linked with poor knowledge regarding antibiotic use and residues (Table 11).

Additionally, this meant that those with higher age especially above 50 years had a better understanding and experience about antibiotic use and residues hence better KAP than those aged below 50 years and on the other hand it implied that males had better KAP scores than females. The findings also showed that among those with lower education levels (none to primary) they had poor knowledge. This clearly shows that education can influence a farmer to have good knowledge, then change their attitudes and promote good practices towards antibiotic use and residues.

During this study it was clearly seen that the level of education among smallholder dairy producers significantly influenced their KAP scores Table 10. This means that education of farmers plays a very essential role to change their KAP towards antibiotic use and residues. It was also seen that the majority of those smallholder dairy producers in Mbala aged 30 to 50 years exhibited poor knowledge (Table 11). On the other hand, the level education (non to primary) among smallholder producers

influenced their KAP, such that it was observed that majority of those respondents with poor KAP had lower educational levels (especially those from none to primary) (Table 11).

The findings of this study were compared with other studies that reported on KAP among dairy producers. In contrast to this study, the findings in a study in Gweru district, Zimbabwe by Gonah *et al.* (2024) reported that majority of farmers had good knowledge (83 %), positive attitudes (81 %), and good practice (81 %). This means that there is a very higher likelihood of compliance with set regulations with regards to antibiotic use and residues among farmers in Gweru Gonah *et al.* (2024) that farmers reported in this study.

Another study conducted in Ethiopia by Tufa *et al.* (2023) reported that most farmers showed poor knowledge and unfavourable attitudes about antibiotic use and residues which aligned with this study finding. This implies that there could be higher risk of antibiotic misuse and overuse in both studies which results in antibiotic residue deposition in milk above MRLs which is currently a global public health threat. In contrast to the current study, another study in Ethiopia done by Kallu *et al.* (2024) reported more than half of the surveyed dairy farm owners/workers had good knowledge, appropriate practice and desirable attitudes.

Additionally, this study established that those smallholder dairy producers with a less academic background were found to have poor knowledge, negative attitude and poor practices. A study conducted by Ozturk *et al.* (2019) discovered that farmers holding a postgraduate degree had a markedly higher level of knowledge about antibiotics compared to those who graduated from high school, primary school, or had less formal education which aligned with findings of this study. Since majority of smallholder dairy producers had lower educational level, there is need for training to influence understanding and have good KAP amongst themselves to promote prudent antibiotic use.

A study in Sudan Eltayb *et al.* (2012) found that higher levels of education were significantly associated with increased knowledge of AMU, AMR and disease among farmers which aligned with findings of this current study. During this study it was observed that those with higher educational levels had better understanding about antibiotic use and residues as compared to those with lower education. This implies that education plays a very crucial role in promoting good KAP among smallholder dairy producers.

Another study done in Zimbabwe by Gonah *et al.* (2024) among dairy producers reported that knowledge of antibiotic use was associated with a higher level of formal education which align with this study findings. A study by Dankar *et al.* (2022) reported that higher levels of education are associated with improved literacy, which can enhance access to, utilization of, and comprehension of informational and educational resources regarding the use and effects of antimicrobials. Therefore, this greater literacy can help individuals recognize and dispel common myths and misconceptions about antibiotic use which was seen amongst respondents with tertiary education during this study.

The inadequate KAP amongst smallholder dairy producers in this study regarding antibiotic use and residues suggest inconsistent antibiotic usage. This aligns with findings from a study by Dankar *et al.* (2022) in Lebanon, which reported that smaller dairy producers often employed antimicrobials in an unstructured or inconsistent way. During this study, some respondents did not understand of what antibiotics are and their uses and antibiotic residues and this was reported with a significance difference between two districts. This meant that farmers from Kasama knew better of what antibiotics are and their uses and antibiotic residues than those of Mbala.

This study also reported that some participants did not know what is antibiotic withdraw period and how to determine it. This is vital because if a farmer does not understand the withdrawal period and how to determine the antibiotic withdraw period, it may result in misuse and overuse and, ultimately, the presence of antibiotic residues in the milk above MRLs which is public health concern. A total of 100% respondents in a study by Kaneene & Miller, (1997) did not know antibiotic residues which was higher than this study. This implies that farmers in this study might have heard of antibiotic and their residues but do not conform to the required antibiotic use and food safety measures.

The majority of respondents in this study were unknowledgeable about antibiotic withdrawal period, compared to 90% in a study by Mohamed *et al.* (2020) conducted in Benadir, Somalia. In these studies, the level of the knowledge regarding antibiotic withdraw period was lower a situation which might consequently cause antibiotic residues deposition in the milk produced. A survey among dairy producers in Michigan, USA, indicated that lack of understanding of drug withdrawal periods significantly contributed to the presence of drug residues in milk as reported by Kaneene & Ahl (1987), which aligned with the findings of this study.

Njoga *et al.* (2018) established that inadequate adherence to antibiotic withdrawal periods was linked to the low educational levels among farmers, which was consistent with the findings of this study.

Most respondents had lower educational which was correlated with poor KAP, hence lower understanding about antibiotic withdraw period and its application during antibiotic use. The majority believed that giving antibiotics to healthy animals led to faster growth and increased milk production. This perception significantly contributes to antibiotic misuse, driven by the desire for increased milk production and faster growth. This behavioural tendency led to a higher prevalence of antibiotic residues in milk.

A proportion of some respondents thought that antibiotic residues would not be present in milk for consumers if the animals were treated with antibiotics or if the withdrawal period was not observed. These farmers were also not aware of the adverse effect of continuous consumption of antibiotic residues in milk. A study conducted by Beyene (2015) found that farmers lack of awareness regarding the residual effects of ARs in milk on human health, along with inadequate education for farmers, may result in antibiotic deposition which aligned with this study findings. This situation causes leakage of antibiotics residues following violation of regulations for antibiotic usage.

A study by Beyene (2015) also indicated that limited or poor awareness about consequences of misuse or overuse of antimicrobials in ASF encourages unethical administration of antimicrobial drugs which might led to deposition of ARs above MRLs, which also align with this study findings and a possible driver of AMR microorganisms in animals and humans. This study also indicated a significant prevalence of practices such as not screening for antibiotic residues in milk, failing to maintain treatment records for their animals and self-administering of antibiotics is fostering inappropriate use of antibiotics which clearly seen in milk samples with higher antibiotic prevalence.

Some of other practices noticed were that farmers were routinely using antibiotics as a preventive measure, not adhering to the antibiotic withdrawal period and use of antibiotics to boost milk production. These findings are critical as they contribute to the misuse and overuse of antibiotics among smallholder producers, ultimately leading to the accumulation of antibiotics in the milk produced which leads to AMR. Non-compliance with AMU, particularly regarding withdrawal periods and self-administering of antimicrobials, was observed in a study in Lebanon by Dankar *et al.* (2022) which also align with study findings.

In addition, the lack of adherence to antibiotic withdraw period may result in ARs in milk, posing a potential risk factor for the development of AMR. AMR is a serious global public health threat (WHO, 2019). Despite, poor KAP among the dairy farmers the majority in this study reported to be

participating in livestock disease control trainings. This could imply that majority of them did not put into practice what they learnt which is an indication of negative attitude towards antibiotic use and residues or they did not frequently attend training programs being offered or did not attend specialised training programs on AMU and their implication in ASF (milk).

In a study conducted in Gweru district, Zimbabwe by Gonah *et al.* (2024) found that individuals with detected antibiotic residues in their raw milk samples had low scores in knowledge, attitudes, and practices which clearly align with this study. These individuals were more likely to be small-scale farmers who had not received specialized training in dairy farming. Therefore, this is clear indication that dairy producers require regular and practicable specialised trainings on disease control, farm management practices and prudent antibiotic use and residue monitoring in the milk being produced.

The respondents who reported obtaining antibiotics from agro-vet shops in this study, was lower than 83% (Caudell *et al.*, 2020). This meant other respondents sourced antibiotics from veterinary personnel followed by fellow farmers and pharmacies. The practice of sourcing antibiotics from fellow farmers presents another potential pathway for misuse, as there is no regulatory oversight when farmers exchange medications among themselves. This finding align with a study among dairy farmers Dankar *et al.* (2022), which showed that irregular antibiotic use was linked to easy access to antibiotics, disrupted relationships with veterinary, and a lack of self-assessment and inspection.

A study conducted in Somalia, found that 70% of participants did not consult or engage with veterinary official Mohamed *et al.* (2020), which was notably higher than this study finding. This finding could suggest that participants engaged their veterinary staff yet still did not conform to required drug use and withdraw period. This could imply negative attitude among them and weak regulatory controls in obtaining and using of antibiotics among smallholder dairy producers in both districts. Majority of respondents did not keep animal treatment records which align with a study done in Benadir, Somalia by Mohamed *et al.* (2020) which reported 90%.

A survey in Michigan, USA indicated that poor record-keeping significantly contributed to the presence of ARs in milk as reported by Kaneene & Ahl, (1987), which was also consistent with findings of this current study. Kaneene & Miller, (1997) reported that 100% of respondents did not screen for any antibiotic residues in the milk they produced, which was aligning with this current study. Lack of antibiotic screening protocols could potentially lead to the presence of antibiotic drug residues in milk above MRLs which was consistent with the current study findings and may subsequently contribute to the development AMR (Kaneene & Miller, 1997; Kebede *et al.*, 2014).

The participants of this study reported that tick borne diseases, mastitis, diarrhoea associated conditions and lumpy skin disease were the most common conditions and mainly reported during rain and cold season. This implied that majority of these cattle diseases among smallholder dairy producers were experienced during rainy and cold season which could lead to more usage of antibiotics during therapeutic usage to manage these diseases. This somewhat creates a risk of deposition of antibiotic residues in milk being produced during these two seasons, which was clearly evident in the milk samples collected from both districts during cold season.

In addition, most respondents during this study reported using oxytetracycline and sulphonamides which align with a report from Mainda *et al.* (2015), which indicated that oxytetracycline, penicillin's and sulphonamides are the most commonly used antibiotics among livestock farmers. Caudell *et al.* (2020) reported that majority of drugs used among livestock farmers contained 70% tetracycline and 20% macrolide or aminoglycoside. Dankar *et al.* (2022) reported penicillin, neomycin, oxytetracycline, tylosin, sulphonamide's, amoxicillin, florfenicol, and erythromycin as most commonly used drugs among farmers, which was also consistent with the findings of our study.

Majority of commonly used antibiotics among participants in this study were consistent with laboratory findings, which identified sulphonamides and macrolides as the most frequently detected antibiotic residues in milk samples. This indicated their indiscriminate use and lack of adherence to antibiotic withdraw period. Despite, oxytetracycline being commonly reported highest among this study respondents, it was detected in only 12.9% of milk samples. This could perhaps suggest its reduction in use during the period of sample collection. Majority of participants indicated that oxytetracycline was mainly used for tick borne disease which is less prevalent in cold season.

Additionally, this could also indicate that farmers are increasingly turning to alternative drugs, such as macrolides, due to oxytetracycline resistance to many infections following it's widely misuse among livestock farmers. The prevalence of antibiotic class residues in milk samples collected from Mbala and Kasama districts of Northern Province, Zambia is alarming. This indicated a widespread and indiscriminate use of antibiotic drugs in dairy cattle within the study areas, posing a significant public health risk. The presence of poor KAP among smallholder dairy producers regarding antibiotic use and residues could have led to deposition of antibiotics in the milk which they produced.

These concerning results necessitate an urgent intervention strategy to tackle the high prevalence of antibiotic residues in milk above MRLs and the lack of adequate KAP regarding antibiotic use and residues among smallholder dairy producers in both Mbala and Kasama district. The distribution of antimicrobial drugs is uncontrolled due to is poorly regulations of pharmaceuticals in majority of African countries (Ducrot *et al.*, 2021). This implies that there is a higher possibility of antibiotic misuse and overuse in developing countries like Zambia leading to antibiotic deposition in ASF which is among serious public health concerns.

Therefore, this underscores an important need to control antimicrobial drugs in ASF using a “One Health” including education and routine sensitization programs of smallholder dairy producers on the importance of antimicrobial stewardship, adherence to antimicrobial withdraw period and public health risks of antimicrobials in milk and AMR. As part of the global community Zambia adopted the “One Health” collective approach as strategy address AMR (Kapona, 2017). It is thus imperative to enforce a practicable regulatory framework to strengthen monitoring and surveillance of antibiotic residues as described Barros *et al.* (2023) and AMR in milk and other ASF to assure food safety.

Additionally, smallholder dairy producers should implement routine screening for antibiotic residues in milk using practical, cost-effective, and user-friendly tests, such as microbial inhibition tests. These tests can detect a broad range of ARs in a single assessment (Pikkemaat *et al.*, 2009). Some examples of commercially available tests include Delvotest SP, Charm Farm-960, Copan, and others that utilize *Bacillus stearothermophilus var. Calidolactis* spores (Žvirauskiene & Šalomskiene, 2007). These dairy farmers can collectively procure such rapid test and acquire capacity buildings through the District Veterinary Office and the cooperatives they belong too.

Furthermore, there is need for provision of readily available veterinary officials to livestock farmers and enhance awareness about antimicrobials stewardship and concerns about antimicrobials in ASF and AMR and its distribution pathways among policymakers, smallholder farmers, veterinary staff, public and relevant stakeholders. A study by Njoga *et al.* (2018) emphasized the importance of raising awareness and encouraging farmers to implement good farm management practices, such as proper nutrition, routine vaccinations, good hygiene, and biosecurity measures, rather than heavily relying on prophylactic antimicrobial administration in food animals to control diseases.

The study was just limited to qualitative analysis of antibiotic residues in milk from two districts in Northern Province, Zambia. Additional studies should assess the quantities of antibiotic residues and

AMR across different ASF in Zambia. There is also need to conduct assessment of KAP on other livestock farmer about antibiotic use, residues and AMR in several regions of the country and provide a better national outlook.

## **8. CONCLUSION AND RECOMMENDATIONS**

In conclusion, this study managed to assess the presence of five (5) class of antibiotic residues in milk. This study highlighted an alarming presence of antibiotic class residues in the milk samples, with a significant difference between the districts. Mbala presented higher proportion of positive milk samples than Kasama. Sulphonamides and macrolides were the most prevalent antibiotic residues. Only sulphonamides among all detected antibiotic residues had a significant difference between the two districts. It is also concluded that smallholder dairy producers had poor knowledge, negative attitude and poor practices regarding antibiotic use and residues, with no significant difference.

These findings implied indiscriminate use and management of antibiotics, a lack of awareness about their implications, failure to adhere to withdrawal periods, and poor regulatory practices among smallholder dairy farmers. Generally, this situation poses a serious public health risk to consumers within the country. Consequently, there is need for establishing a stringent regulatory framework for antimicrobial drug use among livestock producers, along with routine surveillance and monitoring of ARs and AMR in ASF.

### **Immediate Recommendations**

#### **1. Establish a Regulatory Framework**

Create and enforce strict regulations controlling the use of antimicrobial drugs by livestock farmers.

#### **2. Routine Surveillance and Monitoring**

Start monitoring milk samples for antimicrobial resistance (AMR) and antimicrobial residues (ARs) right away.

#### **3. Educate Farmers on Antimicrobial Stewardship**

Organize urgent workshops or information sessions for farmers about the risks of exceeding Maximum Residue Limits (MRLs) and proper antimicrobial use.

### **Mid-Term Recommendations**

#### **1. Educating Livestock and Veterinary Personnel**

Provide veterinarian and livestock staff with training on the hazards associated with ARs and AMR as well as risk mitigation techniques.

2. Encourage ethical methods of animal husbandry

Promote and assist livestock management strategies that prioritize disease prevention and control in order to reduce the need for antibiotics.

3. Education Programs Regarding Drug Withdrawal Times

Launch educational initiatives emphasizing the value of following prescribed drug withdrawal schedules.

### **Long-Term Recommendations**

1. Employment of veterinary and livestock staff

Employ enough veterinary and livestock personnel to lower the officer-to-farmer ratio. This will improve the amount of time that staff and farmers spend interacting when providing extension services.

2. Continuous Learning and Developing Capabilities

Provide livestock farmers with ongoing education initiatives to bolster their understanding of the hazards to public health and responsible use of antibiotics.

3. Enhance Farm Management Strategies

Encourage enduring collaborations between livestock producers and veterinary services to create sustainable farm management plans.

4. Monitoring and Policy Evolution

AMR and AR trends should be regularly monitored, and laws and rules should be updated in light of fresh data and developments in veterinary medicine.

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## APPENDICES

### Appendix I: Information Sheet and Informed Consent Form



#### FACULTY OF VETERINARY SCIENCE

**Study title:** Antibiotic residues in raw cow milk among smallholder dairy producers in Kasama and Mbala, Northern Province, Zambia.

**Principle Investigator:** Goliath Eneya Zulu

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#### **PART I: Information Sheet**

##### **Introduction**

This research is being conducted by Goliath Eneya Zulu, who is pursuing a Master's degree in Food Safety at Eduardo Mondlane University in Maputo, Mozambique. It aims to fulfil the requirements for the Master in Food Safety degree. The research focuses on assessing antibiotic residues in raw cow milk produced by smallholder farmers in the specified districts as well as evaluating their level of knowledge, attitude and practices regarding antibiotic use and residues. Antibiotic residues are a significant contributor to antimicrobial resistance (AMR) in both humans and animals, representing a pressing global public health threat.

Additionally, I would like to invite you to be part of this important research. You can follow along using the copy provided and feel free to ask any questions at any single time. After all your questions have been answered, you can choose whether or not to participate in this research. Please note that participation is entirely voluntary, and there is no pressure to decide today. You are encouraged to discuss this study with anyone you feel comfortable with before making your decision.

## **Purpose of the research**

Antimicrobial residues (ARs) in animal source foods (ASF) are of global public health challenge. There is currently limited data and surveillance protocols in developing countries especially Africa and Zambia in particular. These antibiotic residues are not often monitored in ASF therefore; humans and animals are at risk of developing AMR. Indiscriminate antibiotic use, poor regulations, lack of knowledge towards antibiotic use and poor management practices in animal production has resulted into deposition of antibiotic residues in ASF. They have been reported cases of human deaths attributed to and associated with AMR.

Zambia reported that standard infections were becoming increasingly difficult to treat with standard first-line antibiotics because of AMR. The reason for this study is to determine antibiotic residues in milk produced among smallholder dairy farmers. We also want to assess the level of knowledge, attitude and practice among dairy producers, towards antibiotic use in Kasama and Mbala districts. The knowledge from this research will help in the adoption of practicable surveillance and monitoring protocols for antibiotic residues in animal source foods. In addition, the outcomes from this research will promote a behavioural change through trainings, education and communication programmes to assure better management practices towards antibiotic use.

## **Participant selection**

Participants in this study will include randomly selected smallholder dairy producers that are currently producing milk and those whose animals are on dry period. The study will not include dairy producers who do not satisfy the aforementioned inclusion criteria. You have been asked to participate in this study because you fit these descriptions.

## **Voluntary participation**

Your participation in this research is voluntary and as such you are free to participate or not. Whether you choose to participate or not, you will still receive all the services offered by the district fisheries and livestock ministerial office and other service providers. In an event you decide to change your mind, you are still free to not take part even if you agreed in the first place.

## **Procedures**

In an event you agree to participate, we will ask you to take part in an interview using our close ended questionnaire which has 3 sections namely; demographic/social data, epidemiological data and

assessment of levels of awareness and knowledge towards antibiotic use. We will also ask you to allow us to collect a milk sample from your milking animals. This will be done after a private discussion with our team and your veterinary personnel and agreeing to take part in the research.

Kindly take note that your name will not be included on the questionnaire and sampling containers, instead they will be replaced with numbers.

### **Risks/ Discomforts**

We do not expect any risks however; if there may be some discomfort during disclosure of information our team will have your local veterinary assistant to help minimize this. The interviewer will work with your local veterinary assistant to help you understand and go through the study. Once you agree, a questionnaire will be administered and then collect a milk sample in a sterile bottle. We would like to assure you that the information we get from you is strictly confidential and will not be shared with anyone outside the research team.

### **Benefits**

The only direct benefit is that if you a farmer have any challenge with your livestock enterprise such as disease, nutrition or any other condition, a local veterinary assistant within our team will be able to make direct follow up and manage the case. The indirect benefits of this study include; information obtained after determination of antibiotic residue in milk and assessment of knowledge towards antibiotic use, will provide scientific evidence-based information required for policy makers and stakeholder to implement monitoring and surveillance protocols that assure food safety to consumers.

In addition, livestock farmers ought to benefit from trainings, education and communication programmes as a way to promote behavioural change and enhance rational antibiotic use.

### **Payments**

During the study there will be no any money being given for your participation in this study. Following, participation in the study any farmer that has challenges regarding production will have an advantage of reporting the matter to their local veterinary staff directly and physically. The local veterinary assistant will be able to make follow ups and help manage the problem. The results from this study will add up the much-needed information and help the policy makers implement measures for antibiotic monitoring in foods of animal origin.

The District Veterinary Office will also be well informed with the outcomes of the research. The office will use this information to promote good management practices among livestock farmers.

### **Confidentiality**

The information that will be collected in this research will be purely kept confidential. This information will be protected from public consumption and will only be accessed by the researchers for a noble cause. During this research no names will be put on any information collected from you but instead, we will use numbers for identification. Be assured that the allocated number will not be shared with or given to anyone except the District Veterinary Officer in case your animals are suspected of a notifiable disease. As for the milk sample collected, it will bear the number assigned to you and other details. This specimen will be destroyed as soon as the study is completed.

### **Sharing the results**

The knowledge acquired from conducting this study will be shared to the Ministry of Fisheries and Livestock that shall transmit the findings to you through your District veterinary officers and Veterinary assistants. After that, we will publish the results so that other interested people or researchers may learn from our research and utilise the information.

### **Right to refuse/ withdraw**

This study allows you to feel free to whether participate in this study or not. The study also allows you to feel free to leave at any point during the interview. You are also free not to answer any questions that you are not comfortable with. You are also free not to allow us to collect a milk sample from your farm. You must be rest assured that your choice not to participate in this study will not disadvantage you from any kind of service provision. Kindly take note that your choice and rights will be well respected.

### **Who to contact**

If you have any available questions, you may ask them now, at any time or during the study. If you wish to ask questions later, you may contact any of the following:

- The principal investigator (Supervisor), Goliath Eneya Zulu on +260976744511 (if you have any questions or complaints as a result of being in this study)

- You may also contact DISTRICT VETERINARY OFFICE in Kasama: Mr Sindamu Mungoni, +260 771958 062 and Mbala district: Mr Brian Mulasu, +260 977 253 338 (if you have any questions or concerns about the study).
  
- You may also contact DIRECTOR VETERINARY SERVICES, Ministry of Fisheries and Livestock, Headquarters, Mulungushi House, Lusaka, Zambia (if you have any questions or concerns about the study).

**PART II: Informed Consent Form**

I have read the provided information or the information has been clearly read to me, and I fully understand it. I have been given the opportunity to ask questions, which have been answered to my satisfaction. I voluntarily agree to participate in this research, and my informed consent will be given verbally.

**PART III: Statement by the Researcher/ person taking consent**

I have accurately read out the information sheet to the potential participant, and to the best of my knowledge and ability I made sure that the participant understands that the following will be done:

1. Interview will be conducted using a close ended questionnaire on the farm.
2. Milk sample will be collected from the participant’s farm.

I confirm that the participant was given adequate chance to ask questions about the study and all the questions asked by the participant have been answered correctly and to the best of my knowledge and ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

**Name of Researcher/ person taking the consent** \_\_\_\_\_

## Appendix II: Questionnaire for Human survey

Questionnaire Serial No \_\_\_\_\_

Topic: A survey conducted to assess the levels knowledge, attitude and practice about antibiotic usage among smallholder dairy producers in Kasama and Mbala Districts, Northern, Zambia.

*Please note that all the information will be treated as confidential.*

### General information

Name of interviewer: \_\_\_\_\_ GPS: \_\_\_\_\_

Participant's ID: \_\_\_\_\_ Cooperative name if belong to any \_\_\_\_\_

Name of district: \_\_\_\_\_ Camp name: \_\_\_\_\_ Village: \_\_\_\_\_

### Section A: Demographic/social data

1. How old are you? \_\_\_\_\_
2. Farm owner's sex? Male: \_\_\_\_\_ Female: \_\_\_\_\_
3. What is the highest level of education attained?
  - a) None \_\_\_\_\_
  - b) Primary \_\_\_\_\_
  - c) Secondary \_\_\_\_\_
  - d) Tertiary \_\_\_\_\_
4. Are you in gainful employment? (If yes please specify)
  - a) Yes \_\_\_\_\_ Specify \_\_\_\_\_
  - b) No \_\_\_\_\_
5. How long have you been a dairy farmer?
  - A. Less than 1year \_\_\_\_\_
  - B. Above 1 to 5 years \_\_\_\_\_
  - C. Above 5 to10 years \_\_\_\_\_
  - D. Above 10years \_\_\_\_\_
6. What is the reason for dairy farming?

- A. Consumption
- B. Sale
- C. Consumption and sale

7. What is your monthly income?
- a. Less than K500 \_\_\_\_\_
  - b. Above K500 to K1000 \_\_\_\_\_
  - c. Above K1000 to K5000 \_\_\_\_\_
  - d. Above K5000 \_\_\_\_\_

**Section B: Epidemiological data**

8. What type of livestock is kept on the farm?
- a) Dairy cattle \_\_\_\_\_ & Beef cattle \_\_\_\_\_
  - b) Sheep \_\_\_\_\_
  - c) Goats \_\_\_\_\_
  - d) Pigs \_\_\_\_\_
  - e) Poultry \_\_\_\_\_
9. What is the total number of animals?
- a) Dairy cattle \_\_\_\_\_ & Beef cattle \_\_\_\_\_
  - b) Sheep \_\_\_\_\_
  - c) Goats \_\_\_\_\_
  - d) Pigs \_\_\_\_\_
  - e) Poultry \_\_\_\_\_
10. What is the type of farming system?
- a) Commercial \_\_\_\_\_
  - b) Medium-scale \_\_\_\_\_
  - c) Small-scale \_\_\_\_\_
11. What is the type of husbandry used?
- a) Intensive \_\_\_\_\_
  - b) Semi- intensive \_\_\_\_\_
  - c) Extensive \_\_\_\_\_
12. How many dairy cattle do you have? \_\_\_\_\_

13. How many dairy cattle produce milk? \_\_\_\_\_

14. How many litters on milk do you produce dairy? \_\_\_\_\_

15. Do you experience any disease condition with your dairy cattle?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

16. What type of common disease conditions?

a) Mastitis \_\_\_\_\_

b) Diarrhoea \_\_\_\_\_

c) Tick-borne Diseases (TBDs) \_\_\_\_\_

d) Lumpy skin \_\_\_\_\_

e) Others (Specify) \_\_\_\_\_

17. What time of the year do you often experience cattle diseases?

A. Rainy season \_\_\_\_\_

B. Cold season \_\_\_\_\_

C. Windy season \_\_\_\_\_

D. Hot season \_\_\_\_\_

### **Section C: Knowledge, attitude and practice about antibiotic use and residues**

#### **Knowledge**

18. Do you know what antibiotics are and their use?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

19. Do you know what antibiotic residues are?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

20. Can giving of antibiotics to animals that are not sick make them grow faster and produce more milk?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

21. Should a farmer wait for a period of time before consume animal source foods (milk) when the same animal is on antibiotics?

- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_
22. Can antibiotic residues be passed through milk to consumers when animals producing that milk are on antibiotic treatment?
- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_
23. Can continuous consumption of antibiotic residues in animal source foods cause side effects to humans?
- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_
24. Should a farmer get a prescription from a veterinarian before purchasing an antibiotic?
- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_
25. Do you use antibiotics for disease treatment?
- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_
26. Do you use antibiotics as a prophylactic measure?
- c) Yes \_\_\_\_\_
- d) No \_\_\_\_\_
27. Do you use antibiotics to increase your milk production?
- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_
28. What do you do with the milk when your milking animal is on antibiotics (observing antibiotic withdraw period)?
- a) Sell \_\_\_\_\_
- b) Consume \_\_\_\_\_
- c) Others (Specify) \_\_\_\_\_
29. Do you treat animals on your own when they are sick?
- a) Yes \_\_\_\_\_
- b) No \_\_\_\_\_

30. Do you keep records for animal treatments at your farm?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

31. Do you consult with your area Veterinary assistant for a prescription before obtaining antibiotics?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

32. What is the most common antibiotic used?

a) Oxytetracycline \_\_\_\_\_

b) Sulphadimidine \_\_\_\_\_

c) Amoxicillin \_\_\_\_\_

d) Tyrosine \_\_\_\_\_

e) Streptomycin \_\_\_\_\_

f) Others (specify) \_\_\_\_\_

33. Where do you source antibiotics from?

a) Agrovvet \_\_\_\_\_

b) Pharmacy \_\_\_\_\_

c) Veterinarian \_\_\_\_\_

d) Others (Specify) \_\_\_\_\_

34. Do you check for antibiotic residues in the milk you produce?

a) Yes \_\_\_\_\_

b) No \_\_\_\_\_

***Thank you for your co-operation***

**Appendix III: Milk sample collection sheet**

Province name: \_\_\_\_\_

District name: \_\_\_\_\_

Sample type: \_\_\_\_\_

Species: \_\_\_\_\_

<b>Date of collection</b>	<b>Time of collection</b>	<b>GPS Coordinates</b>	<b>Study ID No.</b>	<b>No. of samples collected</b>	<b>Condition @ collection</b>	<b>Name of officer</b>