HYGIENIC PRACTICES IN MILK HANDLING AND PREVALENCE OF *CAMPYLOBACTER* IN RAW COW MILK SUPPLY CHAINS IN MAYA CITY, OROMIA REGIONAL STATE, ETHIOPIA

MSc THESIS

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APPROVAL SHEET

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As thesis research advisor, I have read and evaluated this thesis prepared under my guidance by **Ishetu Namomsa**(DVM**)**, entitled, **Hygienic Practices in Milk Handling and Prevalence of *Campylobacter* in Raw Cow Milk Supply Chains in Maya City, Oromia Regional State, Ethiopia.** I recommend that it can be submitted as fulfilling the thesis requirement.

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Final approval and acceptance of the thesis is contingent upon the candidate's submission of the final copy of the thesis to the Council of Graduate Studies (CGS) through the Departmental Graduate Committee (DGC) of the candidates' major department.

DEDICATION

This research is dedicated to my beloved mother, Dassatu Adino, whose unchanged love, support, and encouragement have been my guiding light throughout this journey.

STATEMENT OF THE AUTHOR

First of all, I affirm that this thesis is entirely original with proper citations to all sources used in its production. This thesis has been submitted as part of the requirements for an M.Sc. in Veterinary Public Health at Haramaya University. It is housed with the university library and will be made available to borrowers in compliance with library policies. It is acceptable to use brief quotes from this thesis without obtaining permission as long as the source is properly cited. If the head of the major department or the dean of the Directorate of Graduate Studies determines that the proposed usage advances scholarly interests, they may grant requests for permission to use extensive quotations or to reprint the text in whole or in part. In any other situation, the author's consent is required.

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BIOGRAPHICAL SKETCH

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LIST OF ACRONYMYS

AGS	Atmosphere Generation System
CDC	Center For Disease Control
CFU	Colony-Forming Unit
DALYS	Disability-Adjusted Life Years
EFSA	Europe Food Security Agency
FAO	Food And Agriculture Organization
FERG	Food Borne Disease Burden Epidemiology Reference Group
LPS	Lipopolysaccharide
mCCDA	Modified Charcoal Cefoperazone Deoxycholate agar
NCTC	National Collection of Type Cultures
PCR	Polymerase Chain Reaction
qPCR	Real-Time Quantitative PCR
TSA	Tryptone Soya Agar
UI	Uncertainty Interval
VIF	Variable inflation factor
WHO	World Health Organization

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ABSTRACT

Campylobacter is an intestinal bacterium of animals, especially poultry, cattle and other livestock. It is one of the most common bacterial causes of foodborne infection in humans worldwide, usually through the ingestion of contaminated food and water. The aim of the study was to estimate the prevalence of Campylobacter in raw cow milk through its supply chain and to assess the contribution factors for contamination of raw cow milk in Maya City. A total of 127 raw cow milk samples with 60 milk contact associate environmental samples were collected from February 2024 to July 2024 through a cross-sectional type of study. Raw milk and environmental samples were collected and processed using enrichment, differential, and selective medium. Risk factors for Campylobacter occurrences were assessed using culture method laboratory diagnosis of milk and environmental samples and structured questionnaire surveys, followed by binary and multivariable logistic regression analysis. The prevalence of Campylobacter in tested raw cow milk was 11.02% (95% CI: 0.95-9.68), and in environmental samples it was 8.3% (95% CI: 0.14-1.71), resulting in an overall prevalence of 10.70%. The occurrence of Campylobacter in raw cow milk was significantly associated with poor cleanness of the house (AOR: 14.35, 95% CI: 1.25-164.62), poor cow cleanliness (AOR: 5.7, 95% CI: 1.23-40.20), use of unclean containers (AOR: 6.63, 95% CI: 1.28-34.35. The current study indicated that there is a significant prevalence of Campylobacter in fresh farm milk from milk selling sites (17.7%) and udder milk (6.6%) from individual dairy cows. A questionnaire survey revealed limited awareness, with 95% of respondents lacking training in hygienic milk handling. The prevalence of Campylobacter in raw cow milk supplied to the community is slightly high in the Maya City East Hararghe zone, Ethiopia, when compared with other studies in the country. It is highly associated with hygiene practices in the milk supply value chain. Thus, strict hygiene measures, including cleaning and disinfection of milking areas, equipment, and containers, as well as efforts should be made to establish baseline data for Campylobacter prevalence in the study area to enable trend analysis and better epidemiological understanding.

KEYWORDS: *Campylobacter, Hygienic practices, Maya city, Prevalence, Raw cow milk, Risk Factors.*

1. INTRODUCTION

1.1 Background and Justification

Food safety is becoming a more significant issue in terms of human health, especially the safety of items originating from animals. With increase in the consumption of products of animal origin the risk of foodborne diseases of humans also increases. The raw food movement, characterized by commonly distributed in raw form is milk. Raw milk is a body tissue growth and maintenance promoter through contribution of the required nutrient ingredients, such as proteins, energy, minerals, and vitamins. It is also a vehicle and medium for notorious pathogens, such as Mycobacterium *bovis*, E. *coli*, Listeria *monocytogenes*, *Campylobacter*, *Brucella*, and *Salmonella* (Zenu and Bekele, 2023).

The genus *Campylobacter* contains 32 species and 9 subspecies (Costa and Iraola, 2019).And the majority of *Campylobacter* species are microaerophilic and need reduced oxygen (3-10%) again raised CO2 (5-10%) levels (Huang and Garcia, 2022). C. *jejuni* is the major frequently reported *Campylobacter* species (80% to 90%) followed by C. *coli* (5% to 10%) (Levallois *et al.*, 2014).*Campylobacter* is sensitive to freezing and drying but it is death rate is dependent on temperature. They can survive at refrigeration temperatures (4°C) and in meat stored frozen (at -18°C to -22°C) for several weeks (Wagenaar *et al*., 2013).

Its species are known to cause gastroenteritis in human, caused pre dominantly by C. *jejuni*, followed by C. *coli*, C. *lari* and C. *fetus. Campylobacter* is remained as global health concern as the group *Campylobacter* has zoonotic potential, large range of reservoir hosts, and environmental persistence (Masila *et al.,* 2020). Human gastroenteritis caused by *Campylobacter* can range in severity from moderate to severe diarrheal illness. After being exposed to the bacterium, symptoms usually last one week and include fever, cramping, and abdominal pain in addition to diarrhea (usually bloody diarrhea). Meningitis, reactive arthritis, hemolytic uraemic syndrome, and Guillain-Barre syndrome are among the complications caused on by *Campylobacter*. Campylobacteriosis outbreaks have repeatedly been associated with the consumption of raw milk (Bandick *et al.,* 2023).And also, other contaminated drinking water, direct contact with animals,

fecal runoff of domestic animals and especially chickens, and contaminating surface water act as the main source of organisms (Asuming-Bediako *et al.*, 2019).

Campylobacter species can cause abortions, enteritis, and infertility in various species of animals. In chicken C.*jejuni* infection lead to prolonged inflammation, damage of the gut, and diarrhea (Humphrey *et al*., 2014). In some laboratory animal species, enteritis caused by C. *jejuni* and rarely C. *coli* is more severe in young animals and can affect cats, sheep, dogs, poultry, and calves. Mucoid diarrhea with specks of blood with or without fever is typical in calves. In sheep C. *fetus* sub species fetus and C. *jejuni* cause stillbirth, abortion, weak lambs, reduction in milk production, prolonged lambing with immunity revival after re-infection (EFSA, 2010).

Thermophilic *Campylobacter* species colonize the intestinal tract of cattle and are shed intermittently with the feces (Bianchini *et al.,* 2014).Therefore, it is assumed that in raw milk, this pathogen mainly originates from fecal cross-contamination during milking. However, it is not clear how this contamination takes place and how often raw milk is contaminated during milking (Giacometti *et al*., 2015). And also Milk may be possibly contaminated by the direct discharge of a mastitis-affected cow or by excrement from diseased or colonized cattle during milking (Anwer *et al.*, 2024). Thermo tolerant *Campylobacter* species colonize the intestinal tracts of a wide range of mammals and birds, usually without causing clinical disease, and are ubiquitous in the natural environment (Humphrey *et al*., 2007).

Many studies have been carried out in various regions of Ethiopia to determine the prevalence of *Campylobacter* in both animal and human populations. A study conducted in the Oromia Region, Ethiopia, reported a prevalence of 9.1% in cow milk (Eshetu *et al.*,2023). Another study conducted in Oromia Region Bishoftu reported a prevalence of 23.7% in humans with gastroenteritis (Teklu *et al*., 2019). While the study conducted in Addis Ababa reported prevalence in cows of 12.9% (Zelalem *et al.,* 2019a). And the study conducted in Urban and Rural Settings of Eastern Ethiopia, reported that *Campylobacter* accounted for 8.4% in Diarrheic Under Five Children and Tracked Human Contacts (Belina *et al.,* 2023). In addition, the study conducted at Jimma Town Abattoir, Ethiopia indicated prevalence of 5.6% (38/684) (Debelo *et al.,* 2022).

1.2 Statement of Problem

Food borne disease is a common problem in developing countries; most infections are food-borne, caused by the consumption of unpasteurized milk, contaminated water, and meat, particularly poultry meat, rather than human-to-human transmission (Abamecha *et al*., 2015). The problem is severe in developing countries like Ethiopia due to limitations in securing optimal hygienic food handling practices. Besides, not much is known about the hygiene practices at farm level, handling of milk, and environmental contamination contributing to the presence of *Campylobacter* (Keba *et al.,* 2020a). Furthermore, it's unclear what risk factors contribute to the contamination of cow milk in Ethiopia and how common it is to find *Campylobacter* species there

Campylobacter is considered an important foodborne pathogen; however, there is little information about its prevalence in raw cow milk in Ethiopia, especially in Maya City and its surroundings. Many of the studies conducted were either on meat products or general zoonotic diseases; thus, there was a need to determine *Campylobacter* contamination in raw milk. This knowledge gap limits the designing of evidence-based interventions that are appropriately fitted to the local perspectives; hence, there is a need for focused research in this area.

This study is crucial in addressing a critical public health problem by identifying the prevalence of *Campylobacter* contamination in dairy products, which are a leading cause of foodborne illnesses. By examining risk factors associated with contamination in dairy cow farms and cow milk selling sites, the study provides essential data to inform targeted interventions aimed at improving milk safety, enhancing hygiene practices, and minimizing health risks to the community. More specifically, the findings add to scientific understanding about transmission dynamics of *Campylobacter* in low-resource settings and provide a foundation for future research and policy developments to ensure food security with public health in Ethiopia and similar regions.

1.3. Objectives of the Study

1.3.1. General Objective

The general objective of the study was, to assess the occurrence of *Campylobacter* in milk and its possible risk factors at different dairy cow farms and cow milk selling points in Maya city, east Hararghe zone, Ethiopia.

1.3.2. Specific Objectives

The specific objectives of these study was:

- To isolate and identify *Campylobacter* from raw cow milk along the dairy cow farms and associated environmental samples from Maya city, east Hararghe.
- To identify potential risk factors associated with the occurrence of *Campylobacter* in raw cow milk.
- To assess milk handling hygiene practices and their association with *Campylobacter* prevalence in raw cow milk supply chains in Maya City, Oromia Regional State, Ethiopia.
- To assess the perception and attitude of the dairy workers towards hygienic practice associated to milk handling during milking, selling and milk consumption.

2. LITERATURE REVIEW

2.1. Overview of the *Campylobacter*

Members of the genus *Campylobacter* have been recognized as foodborne pathogens and are the leading cause of human gastroenteritis worldwide, being responsible for one of four key global cases of diarrheal diseases (Heredia and García, 2018).The *Campylobacter* species were described (as Vibrio) for the first time 1913 by McFadden and Stockman as a cause of bovine and ovine infertility and abortion. *Campylobacter* was isolated from human samples for the first time in 1938 as a milk-borne outbreak. In the late 1950s, *Campylobacter species* was isolated from blood samples of children with diarrhea. Crucial step was taken in Belgium in the early 1970s when *Campylobacter* were isolated from human faeces (Natsos *et al.*, 2019).

The Greek words "campylos," which means curved, and "baktron," which means rod, are the sources of the term *Campylobacter* (Skirrow, 2006). It is one of the four primary causes of gastroenteritis worldwide, and throughout the past ten years, its prevalence has grown in both industrialized and developing nations. Although C. *jejuni* and, to a lesser extent, C. *coli* account for the great majority of reported. *Campylobacter* are Gram negative, non-spore forming, slender, spiral to curved rod-shaped bacteria that are commonly present in the intestinal tracts of domestic and wild animals (Samie *et al.*, 2007). Its species are classified “thermophilic” since they grow between 37 and 42°C, but incapable of growth below 30°C (absence of cold shock protein genes which play a role in low-temperature adaptation), with an optimum temperature of 41.5°C (Levin, 2007).

Since Campylobacteriosis is a self-limiting illness, antibiotic treatment is generally not recommended. Although antimicrobial treatment may be essential in specific clinical situations, treatment may be complicated due to the emergence of antimicrobial-resistant strains of *Campylobacter* due to widespread use of antimicrobial agents in veterinary and agricultural medicine (Bhunia and Bhunia, 2018).*Campylobacter* are considered commensals in food animals, with poultry being the main reservoir (Mageto *et al*., 2018).Human foodborne *Campylobacter* infections have been found to be primarily caused by consuming contaminated poultry meat and

improper handling of raw chicken. Raw milk or milk products, as well as drinking contaminated water, are other documented human transmission sources (Ouko *et al.*, 2021).

2.2. Characteristic features of *Campylobacter*

Campylobacter, mainly *C. jejuni* is viewed as one of the most well-known reasons of foodborne bacterial diarrheal sickness in people around the globe. It is colonizing the digestive system of numerous wild and household animals and birds, particularly chickens. Intestinal colonization brings about transporter/carrier healthy animal (Ammar *et al*., 2021).While one typing approach based on heat-labile antigen has identified 100 serotypes of *Campylobacter* species, another based on heat-stable lipopolysaccharide (LPS) O-antigenic components has differentiated C. *coli,* C. *lari*, and *C. jejuni* into 60 serotypes (Bhunia and Bhunia, 2018). It's critical to describe the pathogenicity markers in strains identified in food for the protection of consumers. The expression of pathogenicity has been attributed to a few potential virulence genes found in *C. jejuni* (García-Sánchez *et al*., 2018).

Members of the genus *Campylobacter* are Gram-negative bacilli. That are tiny, slender, and spirally coiled; they do not generate spores. A *Campylobacter* bacterium is between 0.2 and 0.9 μm wide and between 0.5 and 5.0 μm long. *Campylobacter* can appear as gull-winged or S-shaped organisms and can be seen in chains or pairs. Their gullwing allows them to move quickly. The presence of solitary, unsheathed polar flagella at one or both ends of the bacterial cell gives the genus *Campylobacter* its characteristically quick and darting corkscrew movement as observed by phase contrast microscopy. C. *gracilis* is the sole nonmotile species among all known species. whereas C. *showae* appears as straight bacilli. because of the emergence of many flagella (Silva *et al*., 2018).

Campylobacter species are successful foodborne bacteria and they require complex growth requirements which make them quite fastidious microorganisms (Bhunia and Bhunia, 2018). Since *Campylobacter* are mostly microaerophilic and have a low oxygen need, they can be destroyed by the 20% oxygen present in the atmosphere. These standards make it challenging to diagnose cases of Campylobacteriosis (Hu, 2018).The ideal requirement for *Campylobacter* growth is the microaerophilic states of 3-10% CO2, 3-15% O2 and 85% N2 (Goni *et al.*, 2017).

2.3. Source of infection and Tranismission of *Campylobacter*

2.3.1. In animals

In animals, horizontal transmission of *Campylobacter* often occurs when the organism is consumed by contaminated water, feed, dirt, or fomites (Little *et al.,* 2010). While "pseudo-vertical transmission" via fecal contamination of shells can happen from parent flocks to their hens, vertical transmission from an infected bird to its progeny before shell formation is uncommon. In cattle, C. *fetus* subspecies venerealis is spread venereally (Newell *et al.,* 2011). The pathogen can enter milk through a variety of routes, including animal feces, the milker's hand during hand milking, and the equipment used for milk collection and storage. For example, the pathogen may enter the milk if the dairy cow has mastitis, a mammary gland infection, or a systemic infection (Salihu *et al.,* 2010).

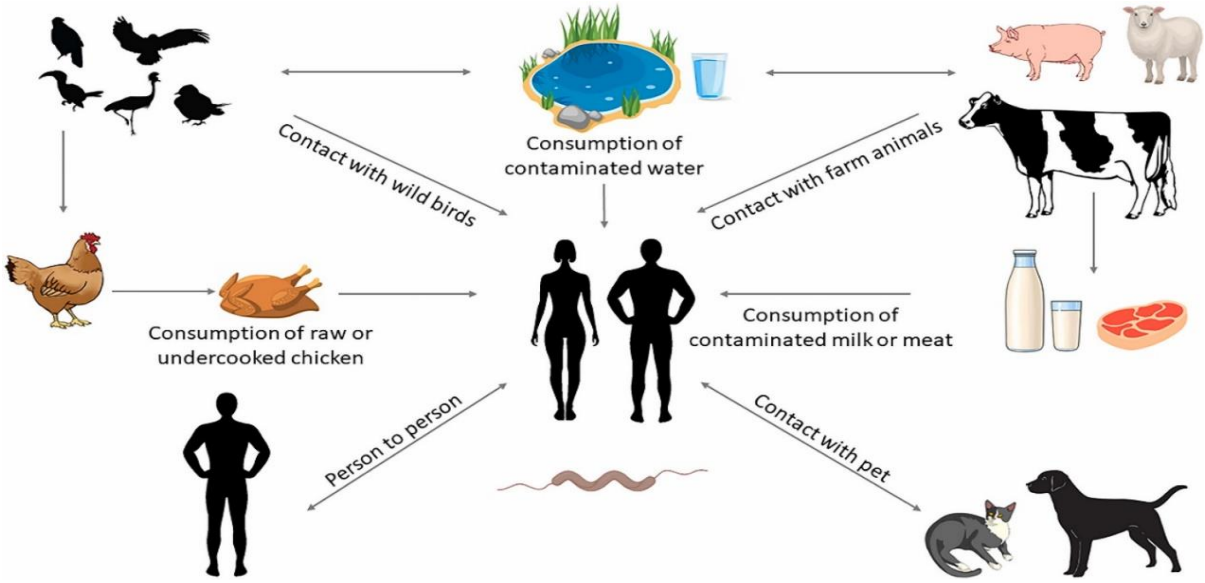
2.3.2. In Humans

Consuming raw or undercooked contaminated food, drinking contaminated water, coming into touch with wild or domesticated animals, or spreading the infection from person to person (fecal-oral or via fomites) are all ways that the microbe might infect humans (Lopes *et al*., 2021).The organism is often found in the caecum of poultry as well as the guts of various domestic animals, including pigs, sheep, cattle, cats, and dogs. Because of their high body temperatures, avian species poultry in particular have emerged as the most well-known reservoir of *Campylobacter* species, accounting for an estimated 80% of human *Campylobacter* infections (Whiley *et al*., 2013). Therefore, the gut mucosa of birds and mammals is thought to be the best place for bacteria to multiply and act as a natural reservoir for *Campylobacter* species. It is a commensal microbe that is present almost everywhere in the digestive tracts of various mammals, making it a ubiquitous microorganism (Llarena, 2015).

Infections with *Campylobacter* can also spread from person to person through fecal-oral or fomites, contaminated food and water, and consumption of unpasteurized milk (Ellis-Iversen *et al*., 2012). Ingestion of infected water, feed, soil, or fomites is the typical horizontal route of *Campylobacter* infection in animals (Little *et al*., 2010). Only in certain cases was the infectious agent isolated from the food in Campylobacteriosis outbreaks linked to raw milk or dairy products

made from unpasteurized milk (Davis *et al.*, 2016). Humans may contract *Campylobacter* species by the consumption of contaminated food and water or through contact with carrier animals (fecal-oral route). Pets in the home are another way that *Campylobacter* infections can spread (Pintar *et al*., 2015).

Figure 1.Reservoirs and routes of transmission associated with Campylobacter species



Source:(Lopes *et al.*, 2021).

Raw milk is a frequent vehicle for transmission of thermophilic *Campylobacter* leading to reported outbreaks. Milk is a challenging food matrix for pathogen detection, due to its high protein and lipid content. Limited detection of *Campylobacter* colony-forming unit (CFU) in raw milk might underestimate the pathogen's infectious potential (Wulsten *et al.*, 2020).

2.4. Epidemiology

2.4.1. Host range

Campylobacteriosis is widespread worldwide colonizing all warm-blooded animals including human beings (CDC, 2013). *Campylobacter* species are a part of the gut flora in many domestic and wild animals. Warm-blooded farm animals such as poultry, pigs, cattle and sheep are major reservoirs and source infection for human being. *Campylobacter* have also been found among wild

birds, wild animals and even in non-vertebrate vectors such as flies. The majority of human infections are caused by C. jejuni (80-90%) and C. coli (5- 10%). C. jejuni is the most prevalent *Campylobacter* found in most animals, with the exception for pigs for which C. coli (Levallois *et al.*, 2014).

Although sporadic *Campylobacter* species infections are most commonly associated with poultry, outbreaks have also been attributed to the consumption of other foods, particularly unpasteurized or raw milk. Based on a review of CDC data from 1997 to 2008, 28% of foodborne *Campylobacter* outbreaks (defined as 2 or more affected people) were due to the consumption of contaminated raw milk products, compared with 11% due to the consumption of poultry (Taylor *et al*., 2013).

2.4.2. Distribution of *Campylobacter* in Animal and Human Beings in Ethiopia

Campylobacter is known to be distributed throughout the world (CR, 2000). Several studies have been conducted in different parts of Ethiopia to investigate the prevalence of *Campylobacter* in animal and human populations. A study conducted in Addis Ababa reported a prevalence of 12.9% in cows (Zelalem *et al*., 2019).

Table 1. Prevalence of *Campylobacter* in raw cow milk and human in different Ethiopia areas

Study area	**Reference**	Source of sample	**Prevalence (%)**
Addis Ababa	(Zelalem *et al.*, 2019)	Animal	12.9%
Jimma	(Debelo *et al*., 2021)	Animal	5.6%
Ethiopia	(Zenebe *et al*., 2020)	Animal	26.2%
Oromia Region	(Eshetu *et al.*,2023)	Raw Cow milk	9.1%
Ethiopia	(Admasie *et al*., 2023)	Raw Cow milk	16%
Eastern Ethiopia	(Belina *et al*., 2023)	Human	8.4%
Jimma zone, South West Ethiopia.	(Gume *et al.*, 2023)	Raw Cow milk	1.3%

There is quite epidemiological difference between low, middle and high income countries which likely arise from differences in diagnostic techniques, biocontrol protocols, food practices, nutritional status, environmental hygiene, climatic condition and the abundance of natural reservoirs (Kaakoush *et al.*, 2015). *Campylobacter* infection are sporadic in nature and have worldwide occurrence (Rautelin and Hanninen, 2000). In UK, it is the principal cause of gastroenteritis while in United States; it is fifth domestically acquired foodborne infection (CDC, 2011).

2.5. Clinical Manifestation of *Campylobacter*

2.5.1. In Animals

Many different animal species can carry *Campylobacter* species asymptomatically (Komba *et al.*, 2013). According to certain research, experimentally inoculating young chickens and turkey poults with *Campylobacter* might result in clinical illnesses such bloody or mucoid diarrhea, weight loss, or even mortality (Zhang and Sahin, 2020). Cattle, sheep, and swine are also colonized by *Campylobacter* species and clinical symptoms in young animals could be more severe than those in adults (Mendonca *et al.*, 2015). *C. jejuni* can cause bovine mastitis and enteritis in calves, whereas *C. fetus* causes abortion and infertility in cattle (Stanley and Jones, 2003).

2.5.2. In Human beings

Campylobacteriosis is the leading bacterial gastrointestinal disease internationally, contributing significantly to the enteric illness burden (Christidis *et al.*, 2016)**.** Inhuman being, the gastroenteritis due to *Campylobacter* ranges from mild to severe diarrheal disease. Instead of diarrhea (often bloody diarrhea), other symptoms are cramping, abdominal pain and fever within 2-5 days after exposure to the organism, with symptoms typically lasting one week (Murray *et al.*, 2006). Complications that occur due to *Campylobacter* are Guillain-Barre syndrome reactive arthritis, hemolytic uraemic syndrome and meningitis, *etc*. (Kopyta and Wardak, 2008). *Campylobacter* causes an acute self-limited disease characterized by diarrhea, fever and abdominal cramps (Keba *et al.*, 2020b).

2.6.Publich Health Significance and Economic Impacts of *Campylobacter*

Campylobacter species have usual considerable attention in recent years as the main cause of bacterial enteritis in human. *Campylobacter* enteritis, considered as an important source of diarrheal illness worldwide. The pathogen is also the main causative agent of 'traveler diarrhea' accompanied by predisposing debilitating factors such as premature birth, pregnancy, chronic alcoholism, cardiovascular disease and neoplasia (Mandrell and Miller, 2006). human *Campylobacter* infection is the third most common reason of mortality between the foodborne microorganisms and death can occurs in immunocompromised patients suffering from liver diseases, cancer and acquired immunodeficiency syndrome (Bhunia, 2018).

Foodborne illness has been estimated to account for US$90 billion annually (Scharff, 2020)**.** WHO's Foodborne Disease Burden Epidemiology Reference Group (FERG) conducts the most thorough evaluation of the worldwide burden of Campylobacteriosis (Havelaar *et al*., 2015). According to FERG, *Campylobacter* caused 31,700 (95% UI 25,400–40,200) instances of GBS and 166 million [95% UI 92–301 million] diarrheal illnesses in 2010 (out of about 2 billion attributable diarrheal illnesses). 37,600 fatalities (95% UI 27,700–55,100) and 3.7 million DALYs (95% UI 2.9–5.2 million; or 54 (95% UI 42–77) DALYs/100,000) were caused by these illnesses](Kirk *et al.,* 2015).Food borne transmission was estimated to contribute to 58% (44–69%) of the global disease burden (Hald *et al*., 2016).

2.7. Diagnostic Approaches of *Campylobacter*

Infection with *Campylobacter* remains a serious public health concern. Pathogens *Campylobacter jejuni* and *Campylobacter coli* are frequently spread through food and are responsible for a significant number of sickness cases worldwide each year (Scallan *et al*., 2011). However, diagnosis may be difficult because the organism is difficult to separate, grow, and identify. (M'ikanatha *et al*., 2012). Unpasteurized milk, untreated water, and poultry meat are common food sources for *Campylobacter* (Joensen *et al.,* 2020). When creating detection methods, it is necessary to take into account the "emerging" and re-emerging human pathogenic *Campylobacter* species found in food and the environments in which they are produced. To find the organism in food

samples of animal origin, a number of diagnostic techniques have been developed, including molecular techniques, immunological assays, and culture-based procedures (Hayet *et al.*, 2021).

2.7.1. Culture-Based Methods

Direct plating of sample mainly stool and food sample onto a *Campylobacter* selective medium, followed by incubation at 42°C under micro aerobic conditions for 48 h, has long been considered the "gold standard" for diagnosis of *Campylobacter* (Fitzgerald and Nachamkin, 2015).Today, there is a wide range of plating media, whose selectivity differs from each other's, and usually grouped in two categories; this includes blood-based agar (Preston, Skirrow, Butzler, Campy-cefex agar) and others based on charcoal instead of blood, such as Karmali and modified charcoal cefoperazone deoxycholate agar (mCCDA). The best recommendation for isolation is mCCDA and Karmali agar since *Campylobacter* colonies were easily recognized, despite their poor sensitivity and productivity, especially in food specimens (Chon *et al*., 2011).

Additionally, many formulations of enrichment broths were made, whose Bolton broth and Preston's formula were the most used in culture methods before isolation step, when bacteria are injured or/and when the number of cells expected in samples is small (Repérant *et al.*, 2016). Even though *Campylobacter* colonies on charcoal plates have distinctive features, an inexperienced scientist may find it difficult to identify them due to the dark background. Nonetheless, CCDA provides a simple method for identifying *Campylobacter* species and pollutants for individuals who possess the necessary expertise. *Campylobacter* species typically show up as gray, flat, swarming colonies on CCDA after being incubated at 42 °C for 36–48 hours (Hayet *et al*., 2021). a microscopic examination for morphology (Gram staining), motility, oxidase, catalase test, and growth under different conditions of suspicious colonies of *Campylobacter* were the most acceptable techniques reported in bio typing scheme in order to confirm the genus (Duarte *et al.*, 2016).

2.7.2. Immunological Methods

Serological tests have been performed for long time in clinical diagnostic and epidemiological studies to define the prevalence of serotype related to disease. All serologic techniques have in common, antibody or antigen detection. The extra intestinal invasion of *C. jejuni* pathogenesis

mechanism can cause bacteremia. In that case, and in order to protect its entity, the body produces specific antibodies against *C. jejuni* (O'Hara *et al.*, 2017)

2.7.2.1. Latex agglutination tests

Latex agglutination tests for rapid identification of *Campylobacter* species have been in use for approximately 20 years. The principle behind this test is the use of polyclonal antibodies to detect flagellar or outer membrane proteins. The latex particles are coated with immunoglobulin's that are raised against antigen from several *Campylobacter* species, primarily *C. jejuni, Coli* and *C. lari* (Miller *et al.*, 2008).

2.7.3. Molecular method

Reducing the time taken to confirm the presence or absence of *Campylobacter* species Rapid techniques such as real-time PCR can detect *Campylobacter* from complex samples but blood in enrichment culture can inhibit the PCR reaction, if applied directly to enriched samples (Williams *et al.*, 2009).Polymerase chain reaction (PCR) can be applied in the diagnosis of Campylobacteriosis, using either species-specific or multiplex reactions based on ribosomal 16S gene sequences, and microarray-based identification tests, have been developed (Man *et al.*, 2010). Real-time quantitative PCR (qPCR) assays have an improved speed for test results and provide quantitative information (Kralik and Ricchi, 2017). Other assays employing qPCR allow multiplexing in the amplification of gene targets by using different fluorophores conjugated to the target probes, and a number of qPCR assays are available for the detection of *Campylobacter* species, which are capable of simultaneously detecting C. *jejuni*, C. *coli* and C. *lari* in a single test (Koziel *et al.*, 2013).

2.8.Treatment and Antimicrobial Resistance Status of *Campylobacter*

Most *Campylobacter* human infections are treated with body fluid replacement and maintenance of electrolyte balance, but antimicrobial treatment is required for severe cases, such as a febrile patient with bloody stool. Antibiotic treatment with macrolides is the predominant choice followed by fluoroquinolones (Whitehouse *et al.*, 2018)**.**Treatment with antibiotics for uncomplicated *Campylobacter* infection is rarely indicated. However, antimicrobial resistance to clinically

important drugs used for treatment (especially macrolides and fluoroquinolones) is increasingly reported for *Campylobacters* (Moffatt *et al.*, 2021).*Campylobacter*s also harbor AMR genes with the capability of horizontal transfer between pathogenic and commensal microorganisms, which could lead possibly to the emergence of multi-drug resistant (MDR) microorganisms (Sheppard and Maiden, 2015).

There is growing scientific evidence that the use of antibiotics in food animals, particularly in developed countries, leads to the development of resistant pathogenic bacteria that can reach humans though the food chain (Avrain *et al.*, 2003). Antibiotic resistance in *Campylobacter* has become a growing concern globally, including in Ethiopia. The development of antibiotic-resistant *Campylobacter* strains is a serious public health threat as it reduces the effectiveness of antibiotics in treating infections caused by these bacteria (Eshetu *et al.*,2023).

Even though Campylobacteriosis is a zoonotic infection, it has been shown that the emergence of *Campylobacter* resistance in human clinical samples is connected to antimicrobial resistance found in animals. The inappropriate usages of antibiotics in the veterinary medicine and animal production contribute to the increased antimicrobial resistance and the emergence of multidrug resistance profiles. The indiscriminate use of antibiotics in food animal production has been indicated as a catalyst in the development of resistant foodborne or waterborne *Campylobacter* infecting humans (Whitehouse *et al*., 2018). In addition, antibiotic use in livestock production is common in Ethiopia, which may contribute to the emergence of antibiotic-resistant *Campylobacter* strains (Amenu *et al*., 2019).Moreover, *Campylobacter* with resistance to antimicrobial agents has also been implicated worldwide (Eshetu *et al.*,2023).The use of antimicrobial agents in dairy cows has resulted in the emergence and dissemination of antimicrobial-resistant bacteria, including antimicrobial resistant *Campylobacter* , which has a potentially serious impact on food safety in both animal and human health (Tafa *et al*., 2014).

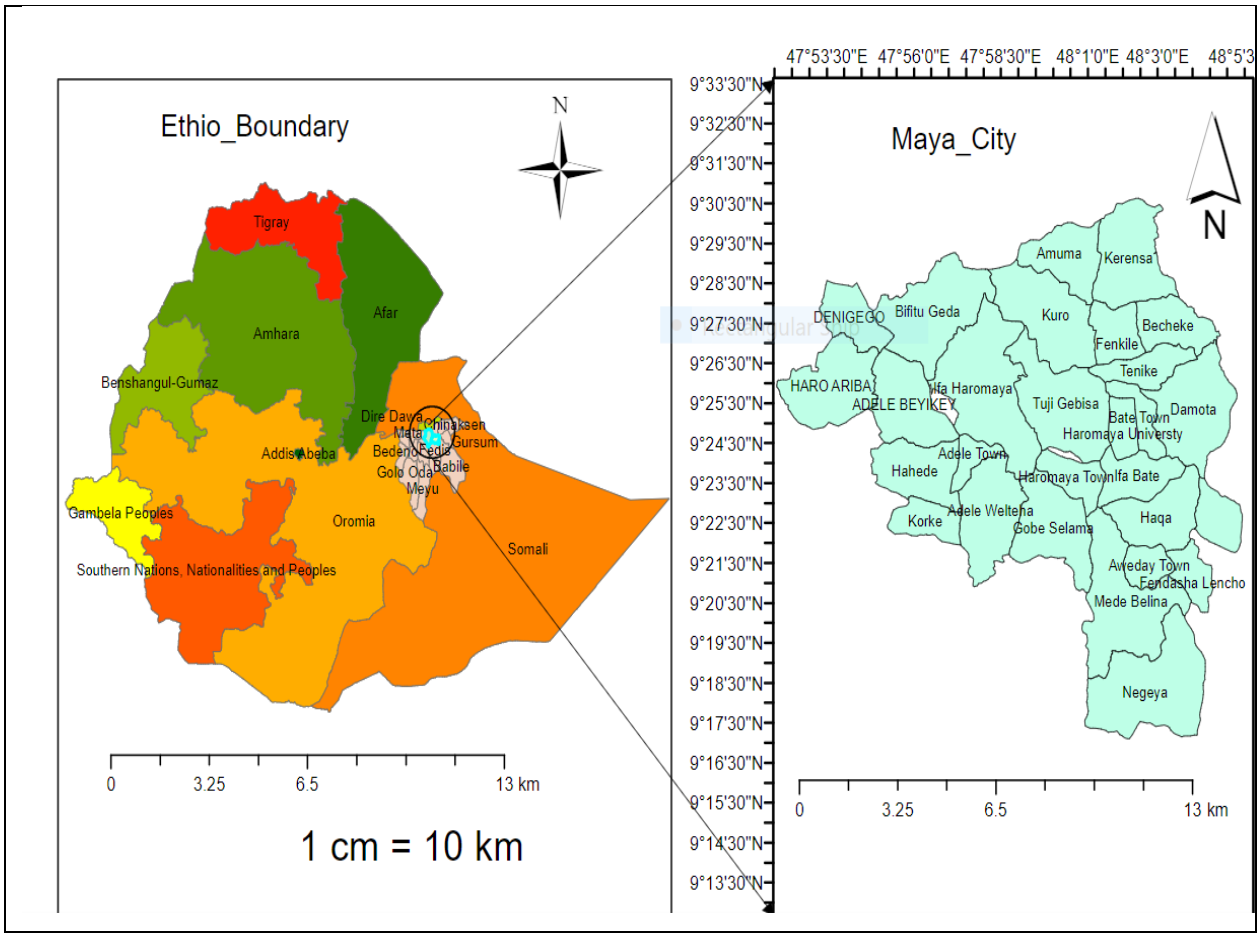
3. MATERIALS AND METHODS.

3.1. Descripion of Study Area

Maya City is situated in the East Hararghe Zone, one of the administrative zones in the Oromia Region, and it is known for its diverse landscape. and agricultural activities. Located approximately 510 km east of Addis Ababa (Capital city of Ethiopia). The elevation of the area is about 2000m above sea level. and geographically it is located at 9° 14' 53" N latitude and 41° 59' 26° E longitude. The area receives 492 mm of average annual rainfall, ranging from 118-866 mm with a bimodal occurrence having short and long rainy seasons that cover February to May and June to September, respectively. The maximum and minimum temperatures are 24°C and 9°C, respectively. The relative humidity of the area is 65%. (MCAB, 2016).

Local, Borana, and Crossbred cattle are reared in and around the study area for milk production. The livelihood in the area is based on agriculture. There are three sub-cities, Haramaya, Awaday, and Adele and sixteen kebeles, which are the smallest administrative divisions. The animal population of Maya City consists of 79,446 cattle, 83,131 goats, 61,830 sheep, 25,000 donkeys, and 100,829 chickens (MCADB, 2023). The area includes dairy farms and a local milk selling site, which serves as an essential center for milk distribution, highlighting the importance of evaluating hygiene practices and contamination risks in the supply chain.

Figure 2.Location Map of Study area (Maya City)



Sources: Arc GIS 10.7.1v Software.

3.2. Study Population

The study population comprised of apparently healthy lactating dairy cows from various dairy farms (including small scale dairy farms) in Maya city. Milk Samples were collected directly from these cows and from fresh farm milk sold at selling points within in the city.

3.3. Study design

A cross sectional study was conducted from February, 2024 to July, 2024 to assess the occurrence of *Campylobacter* in raw cow milk collected directly from udder, milk selling sites and along with as well as associated environmental samples.

3.3.1. Sampling Method and Sample Size Determination

The study was conducted in Maya city, which contains three large-scale dairy cow farms, six main milk selling points, and ninety-two small-scale dairy cow farms owned by farmers (MCADB, 2023). From this Haramaya University dairy farm, Awaday milk selling point, Haramaya Town milk selling point, Haramaya Kebele (01) and Bate Kebele were selected by purposive sampling and simple random sampling were used for individual cows. The milk samples were collected directly from the udder of dairy cows during milking of dairy cows and from milk containers from milk selling points and milk associated environmental samples such as hand swab of dairy workers/milkers, feeding and drinking material surface swab sample and container swab were collected.

The sample size was calculated using the single population proportion formula (Thrusfield, 2005) and was determined based on the following parameters: The desired level of precision, the confidence level, and the expected prevalence of *Campylobacter* from cow milk, which was 9.1%(Eshetu *et al.*,2023).

$$(N) = \frac{Z^2 \times p\,(1-P)}{d^2}$$

$$(1.96)^2 \times 0.091 / (1-0.091) / (0.05)^2 = 127$$

Where N=Sample Size

Z = The standard normal deviation corresponding at 95% of confidence level = 1.96,

P = Prevalence (9.1%= 0.091), Expected prevalence.

d = The degree of accuracy desired (5%= 0.05).

Accordingly, 127 cows milk from Dairy cow's farm and milk selling point found in Maya city and 60 milk contact associative environmental sample were analyzed in this study.

The distribution of milk and environmental samples was intended to confirm a representative assessment of *Campylobacter* contamination across key locations in the milk supply chain. Samples were collected from a dairy farm, milk selling points, and selected kebeles to capture variations in hygiene practices, milk handling, and potential environmental contamination. The allocation of environmental samples was kept uniform across all sites (12 per location) to facilitate a relative analysis of contamination sources. The number of milk samples varied based on the scale of milk production and distribution at each site, ensuring proportional representation and a comprehensive risk assessment

Table 2. Distributions of samples with respect to study sites/address.

Study site	**Sample type**	
	Milk	**Environmental sample**
HU Dairy farm	24	12
Awaday milk selling point	25	12
Haramaya milk selling point	26	12
Haramaya Kebele	23	12
Bate Kebele	29	12

3.3.2. Inclusion and Exclusion Criteria

All farms which actively engaged in dairy production and milk sales were included in the study, irrespective of the cows' breed, age, or production level. In the same way all lactating cows in dairy farms in Maya city during the study period were recruited as target population for sampling. However, lactating cows on antibiotic treatments during the study were excluded. Additionally, any dairy farms whose owners refused to participate were not included in the study.

3.4. Sample Collection and Transportation

Milk sample was collected after cleaning the udder and teats before sample collection when sample was collected directly from udder. To address this, udder and teat Cleaning was performed using a wet towel soaked in disinfectant and clean water or water mixed with disinfectant. Additionally, three or four streams of milk were removed (forest ripped) from the quarter being sampled to minimize the risk of contamination from bacteria at the teat end, while also observing the milk and

mammary quarters for any abnormalities. Observations regarding the hygiene and cleanliness of the milker, udder, teats, equipment, and barn were recorded. The hygiene of the container is considered as good if it appears clean, free from visible dirt, residues, and stains, and is properly washed before use, and it is considered as poor if the container has visible dirt, milk residue, or shows signs of inadequate or no washing. milk samples were collected by direct milking into a sterile 50 ml Falcon tube, as well as from milk containers (utensil) at the milk selling sites.

Environmental sample, i.e., swab samples from milk contact-associated surfaces, including milkers' hands, milk containers surfaces, feeding and drinking material surface swab sample was taken and placed in a 15 ml Falcon tube containing of Bolton broth at each of the selected farms and milk selling points. Hand swab samples were obtained by rubbing the hand surface and the folds between each finger, surface swab and container swab with moistened cotton and coded as from which site it was collected. During sample collection, it was performed properly to avoid contamination of samples, and information such as the site of collection, farm type, farm size, animal ID, parity, that refers to the number of times a cow has given birth to three calves or an equal and cows gave birth for greater than three calves because cows with less than three parities (younger cows) could still be within their peak productive and reproductive years while those with more than three parities (older cows) might have rising susceptibility to infections due to accumulated stress and a generally weakened immune system (Ferreira *et al.,* 2021); (Lean *et al.,* 2023). And type of sample (directly from the udder, milk from container, and environmental sample) was recorded. All samples were aseptically collected in Falcon tubes and individual Falcon tubes containing the samples were placed in stomacher bag (plastic bag) to prevent cross-contamination and immediately transported to the Haramaya University Veterinary Microbiology Laboratory using an icebox containing an ice pack.

3.5. Enrichement,Isolation and Identification of *Campylobacter*

The sample undergone enrichment through the introduction of 10mL of the milk sample into 90 mL of Bolton Selective Enrichment Broth where it was thoroughly homogenized. The prepared sample was incubated under microaerobic conditions achieved using Campygen 2.5 sachet placed in an anaerobic jar with samples. The sample-broth mixture was initially incubated at 37°C for 5hrs and subjected to incubation at 42°C for approximately 44 ± 4 hrs. Following this, the aliquot

was plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) plates, which were incubated once again under microaerophilic conditions at a temperature of 41.5°C for 44±4 hrs.

The growth of *Campylobacter* was detected by its characteristic appearance on the culture media, such as the presence of flat grayish colonies resembling droplets of water sprayed on the medium, or colonies that were creamy or white, moist, flat or slightly raised, extending along the streak line, or regular circular discrete colonies.(Atabay and Corry, 1998).Gram staining was performed to identify the characteristics of *Campylobacter*, which appeared as Gram-negative with an 'S'-shaped and gull-wing morphology. The colonies were then subcultured on TSA at 41.5°C for 44±4 hrs. Additionally, oxidase and catalase tests were conducted for the identification of thermo-tolerant *Campylobacter* genera. Presumptive colonies that tested positive for both catalase and oxidase were preserved as *Campylobacter* isolates. In the laboratory, to identify and reduce errors, positive and negative control strains (*Campylobacter jejuni* NCTC 11351 and *Campylo-bacter coli* NCTC 11351) were used.

3.6. Questionary Survey and Observation

Interviews were conducted with Dairy cow/farm owners, milk sellers, and consumers at different points, and information on the hygiene of the material used for milk storage, the hygiene of the milker, the farming system, and lesions on the teat and udder was collected by observation. The content of the questionnaire also included issues addressing the educational status, health status, personal hygiene, milk handling practices, and food safety knowledge of dairy workers, milk sellers, and consumers. The questionnaire was filled out during face-to-face interviews with them and checklist was also prepared for observational assessment of milk hygiene indicators like level of cleanness (ANNEX II). The questionnaire and observational checklists were managed in accordance with the standard guidelines of the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO and WHO, 2009). The sample size of individuals who participated in the questionnaire was determined using the Harsham Formula, considering a 5% standard error.

The formula used was;

$$N = 0.25/SE^2, \text{ which resulted in } N = 0.25/0.0025 = 100.$$

However, to increase precision, the number was increased to 120.And the survey size was classified to Farm/individual dairy cow holders (76) and milk selling point (44) based on the availability of individuals willing to participate in the study during the data collection period.

3.7. Ethical Review

The study protocol was reviewed and approved by the College of Veterinary Medicine, Haramaya University, and a letter of support was obtained; then official permission was requested from the concerned higher officials of the Maya City Administration and the agricultural office. The consent of dairy workers and the willingness of interviewees were sought to participate in the research. After thoroughly explaining the objectives and relevance of the study, the procedure, benefits, and participants' rights, informed consent was obtained from the participants. It was emphasized that their participation was completely voluntary, that they could choose not to answer any question, and that they could stop the discussion at any time. Participants were also informed that refusing to participate would not affect them or their families in any way and that their responses would remain confidential.

3.8. Data Analysis

All data collected during the study period were checked, coded, and input into an Excel spreadsheet (Microsoft Excel 2010) and subsequently analyzed with STATA software, version 15. Descriptive statistics were used to summarize the raw data. The prevalence was expressed as a percentage, and Pearson's Chi-square test was applied to evaluate the association between risk factors and microbial load occurrence. Univariable and multivariable binary logistic regression analyses were performed to quantify crude and adjusted effects of the risk factors on the occurrence of *Campylobacter* spp. Variables with a P-value less than or equal to 0.25 were selected as potential inputs for inclusion in the multivariable logistic regression analysis in an effort to control for potential confounding variables, and the adjusted odds ratio was calculated, and multicollinearity

was assessed using the variance inflation factor (VIF). Throughout the data analysis, a P-value of less than or equal to 0.05 was considered statistically significant.

4. RESULTS

4.1 Prevalence of *Campylobacter*

In this study, a total of 127 raw cow milk and 60 milk environmental samples were analysed to estimate the rate of *Campylobacter* in cow milk in Maya city. The overall prevalence of *Campylobacter* was 10.16% (19/187) with 11.02% (14/127) in milk sample and 8.3% (5/60) in milk associated environmental samples. The isolation rate of *Campylobacter* 6.6% in udder milk and 17.7% in milk collected from milk selling points (raw, farm-fresh). Notably, the prevalence of *Campylobacter* was significantly higher (P=0.05) in milk samples than in milk associated environmental samples this may be due to environmental factors can be a major cause of milk contamination. **(Table 3)**.

Table 3. Prevalence of *Campylobacter* in cow milk and associated environmental samples

Type of sample	**Sample source**	**Total**	**Prevalence (n %)**	**X^2(P-value)**
Milk sample	Individual cows/udders	76	5(6.6%)	
	Milk selling point	51	9(17.7%)	
Subtotal		**127**	**14(11.02%)**	
Environmental sample	Material Surface swab	20	3(15.0%)	9.1(0.05)
	workers hand swab	20	1(5.0%)	
	Milk Container swab	20	1(5.0%)	
Subtotal		**60**	**5(8.3%)**	
Total		**187**	**19(10.16%)**	

The prevalence of *Campylobacter* in cow milk samples collected from various locations in Maya city was; presented in the following Table**.**

Table 4. Prevalence of *Campylobacter* in cow milk samples by sample source/origin

Sample origin/address	**Frequency**	**Positive**	**Prevalence %**	**X^2(P-value)**
Haramaya University (HU)	24	1	4.2	
Awaday	25	4	16	4.63(0.33)
Haramaya town	26	5	19.2	
Haramaya Kebele (01)	23	1	4.4	
Bate Kebele	29	3	10.3	

Statistical analysis revealed that there was no significant association between *Campylobacter* prevalence and the milk samples collection sites/address (P > 0.05).

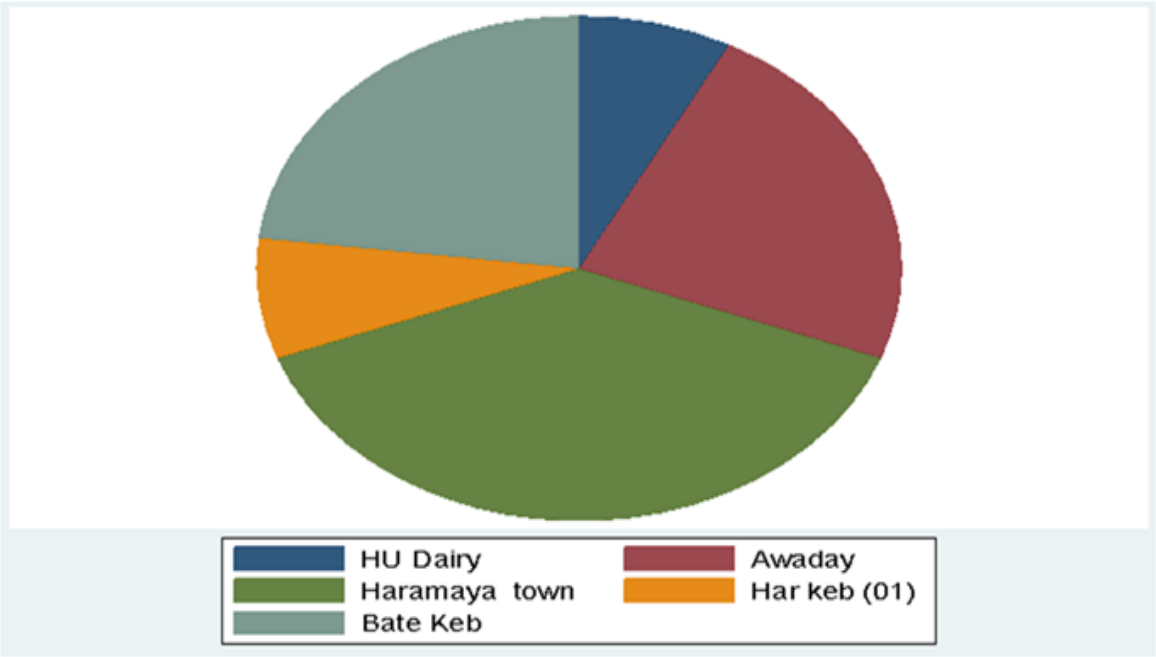


Figure 3.Proportion of sample distribution by address.

4.2 Prevalence of *Campylobacter* and its risk factor at cow level

A total of 76 milk samples were collected from the udders of individual cows. The analysis of cow-level risk factors for Campylobacter contamination showed variable results. Cows that had given birth to fewer than three calves had a higher prevalence, 11.1%, compared to those with more than three calves, 5.9%. Cows in the starting stage of lactation had a higher contamination rate, 10%, compared to those in the mid-to-late stage, 2.8%. Crossbreed and local cows had equal low contamination rates of 5.9% and 6.3%, respectively, while Borana cows showed a slightly higher rate of 10%. Hygiene played an important role: dirty cows had a higher prevalence of 16.7% compared to 3.5% for clean cows. Lastly, cows with udder and teat lesions had a slightly higher prevalence of 9.1% compared to those without lesions, at 6.2%.

Among the variables analyzed for potential risk factors in milk contamination with *Campylobacter* cow cleanliness was the only significant factor. Milk from cows with dirty udder was found to be 5.7 times more likely to contain *Campylobacter* (OR = 5.6; 95% CI: 1.23–36.62).Other factors, such as parity, lactation stage, cow breed, and the presence of udder lesions, did not show a significant association with *Campylobacter* contamination which their P value was >0.05.

Table 5. Logistic regression analysis of cow-level risk factors for *Campylobacter* in raw cow milk

				Univariable logistic regression			Multivariable logistic regression		
Variable	**Categories**	**Animal examined**	**Positives**	**COR**	**95%CI**	**X^2 (P-value)**	**AOR**	**95%CI**	**P-value**
Parity	≤ 3	10	1(11.1)	2	[0.41-36.54]	1.6(0.24)	2.8	[0.19-40.12]	0.75
	> 3†	66	4(5.9)	1	-	-	-	-	-
Lactation stage	beginning	40	4(10.0)	3.8	[0.41-36]	1.6(0.21)	5.4	[0.48-61.01]	0.17
	Mid to last st†	36	1(2.8)	1	-	-	1	-	-
Breed	cross†	34	2(5.9)	1	-	-	-		
	Local	32	2(6.3)	1.1	[0.14-8.05]	0.2(0.95)	-		
	Borana	10	1(10.0)	1.8	[0.14-21.9]	0.2(0.65)	-		
Cow cleanness	Clean†	58	2(3.5)	1	-	-	-	- -	
	Dirty	18	3(16.7)	5.6	[0.85-36.62]	3.9(0.04)	5.7	[1.23-40.20]	0.05
Udder and teat lesion	Absent	65	4(6.2)	1	-	-	-		
	Present	11	1(9.1)	1.5	[0.15-15.10]	0.1(0.72)	-		

❖ AOR= Adjusted odd ratio, COR=Crude odd ratio CI= Confidence interval, X2 =chi-square.

4.3 Prevalence of *Campylobacter* and its risk factor at herd and farm level

Among risk factors at the farm and herd levels, factors such as milking practices, udder washing, animal health status, and having a concrete housing floor, hygiene of milking area showed no statistically significant association ($P > 0.05$) with the occurrence of *Campylobacter*. In contrast, cleanness of the House had a statistically significant association ($P < 0.05$) with the prevalence of *Campylobacter*. Specifically, house cleanliness was significantly associated with the occurrence of *Campylobacter* at the farm and herd levels, with P-values of 0.03 and (CI; 1.25- 164.62). The likelihood of *Campylobacter* being present in raw milk was 14.35 times higher in un clean house than in clean house (OR=14.35, CI=1.25- 164.62)

Table 6. Logistic regression analysis of farm level risk factors for *Campylobacter* in raw cow milk

				Univariable logistic regression			Multivariable logistic regression		
Variable	**Categories**	**Milk examined**	**Positives**	**COR**	**95%CI**	**X^2 (P-value)**	**AOR**	**95%CI**	**P-value**
Milking area hygiene	Good†	67	3(4.5%)	1	-	4.06(0.07)	1	-	
	Poor	9	2(22.2%)	6.09	[0.86-42.9]		19.17	[0.96-268.73]	0.20
Udder Wash	Yes	67	4(6%)	1	-	0.34(0.56)	-		
	No	9	1(11.1%)	1.96	[0.19-19.68]		-		
Milking Practices	By machine†	24	1(4.2%)	1	-	0.33(0.57)	-		
	By hand	52	4(7.7%)	1.9	[0.2-18.13]		-		
Cleanness of the House	Clean†	60	2(3.3 %)	1	-	4.88(0.05)	1	-	
	Dirty	16	3(18.8%)	6.44	[1.01- 44.19]		14.35	[1.25- 164.62]	0.03
Animal Health Status	Good†	64	3(4.7%)	1	-	2.4(0.15)	1	-	0.11
	Poor	12	2(16.7%)	4.06	[0.60-27.46]		6.19	[0.67-57.25]	
floor	Cement†	29	1(3.5%)	1	-	0.75(0.4)	-		
	Floor	47	4(8.5%)	2.6	[0.27-24.52]		-		
Hygiene of milker	Good†	68	4(5.9%)	1	-	0.51(0.48)	-		
	Poor	8	1(12.5%)	2.28	[0.22-23.4]		-		

❖ AOR= Adjusted odd ratio, COR=Crude odd ratio CI= Confidence interval, X2 =chi-square.

4.4 Prevalence of *campylobacter* and its risk factor at at milk selling points

Among of the variables that were analyzed in order to know their effect on the occurrence of *Campylobacter* at milk selling point included the types of containers, the cleanliness of the selling environment, and whether the milk was mixed from different cows and the use of refrigeration indicated no significant difference ($P > 0.05$) in the occurrence of *Campylobacter* in raw cow milk related to these factors. However, the hygiene of the containers was significantly associated with the occurrence of *Campylobacter* in raw cow milk at milk selling point with P value of 0.02 (CI; 1.28-34.35). The odds of having *Campylobacter* in raw cow milk at milk selling points were 6.25 times higher when the containers were poorly hygienic compared to good hygiene (OR=6.25, CI=1.28-34.35).

Table 7. Logistic regression analysis of *Campylobacter* infection in raw milk at milk selling points

				Univariable logistic regression			Multivariable logistic regression		
Variable	**Categories**	**Milk examined**	**Positives**	**COR**	**95%CI**	**X^2(p-value)**	**AOR**	**95%CI**	**P-value**
Type of Containers	Aluminum†	20	2	1	-	1.36(0.31)	-		
	Plastic	23	5	2.5	[0.43-14.6]		-		
	Gourds	8	2	3	[0.34-26.19]		-		
Hygiene of Milk Containers	Good†	39	4	1	-	6.2(0.02)	1	-	0.02
	Poor	12	5	6.25	[1.33-29]		6.63	[1.28-34.35]	
Cleanness of Selling Environment	Good†	31	5	1	-	0.13(0.72)	-		
	Poor	20	4	1.3	[0.3-5.5]		-		
Milk Mixed From Different Cows	Yes	16	3	0.89	[0.19-4.15]	0.019(0.89)			
	No†	35	6	1	-				
Refrigerator used	Yes †	21	1	1	-	3.6(0.05)	1	-	0.09
	No	30	8	6.6	[0.75-57.6]		7.06	[0.73-67.59]	

❖ AOR= Adjusted odd ratio, COR=Crude odd ratio CI= Confidence interval, X2 =chi-square.

4.5.Results of the questionnaire survey.

Sociodemographic characteristics of the respondents

Along with laboratory work, a semi-structured questionnaire survey was conducted with 120 respondents to assess milk handling hygiene practices at the farm, milk selling points and among consumers, as well as their awareness of milk-borne infections. The socio-demographic information of the respondent was included in the next table.

Table 8. Preliminary information of respondents.

Condition	Variables	Frequency	Percent (%)
Address	Urban	26	21.7
	Rural	94	78.3
Sex	Male	28	23.3
	Female	92	76.7
Educational status	No formal education	65	54.2
	Elementary	45	37.50
	High school	6	5.0
	Diploma and above	4	3.3

Of the 120 respondents, 76 were interviewed on their hygienic practices in milk handling at farms and among individual dairy cow holders. Of these, 80.3% cleaned the udder and teats before milking with water, while 19.7% washed with water and then wiped them clean using disinfectant wipes. 90.7% of the respondents are washing their hands after every milking phase, while 9.2% reported washing hands both before starting and after completing milking. Their dairy cow's health status was checked daily, weekly, and when there are signs of illness, by 50%, 34.2%, and 15.8% of respondents, respectively. For storage, 89.5% stored it at room temperature after milking, while 9.2% stored it in refrigerators. On milk transportation, 32.9% transported the milk to the selling point or market immediately after milking, 54% did so a few hours after milking, and 13.2% waited until the end of the day. Visual observation of the hygienic condition of the houses showed that 79% of them were in good conditions, while 21.1% were in poor conditions. The types of

containers used comprise aluminum 56.9%, plastic buckets 30.3%, and other types 13.2% out of 76 observed containers.

Table 9. Practice of respondents related to milk handling at farm and individual dairy cow holder.

Condition considered	**Variable**	**Frequency**	**%**
How do you ensure the cows' udders are clean before milking?	Washing With Water	61	80.3
	Using Disinfectant Wipes.	15	19.7
	Not cleaning	0	0
How often do you wash your hands during the milking process?	After every use	69	90.8
	Before and after	7	9.2
How often do you check the health status of your cows?	Daily	38	50.00
	Weekly	26	34.2
	When There Are Signs Of Illness	12	15.8
How is milk stored after collection?	Refrigeration	7	9.2
	Room temperature	69	89.5
How quickly is milk transported from	Immediately	25	32.9
The farm to the selling point?	Within 2hrs	41	54
	By the end of the day	10	13.2
Animal Housing and Cleanliness	Good	60	79
	Poor	16	21.1
Type of material used to handle milk	Aluminum	43	56.6
	Plastic	23	30.3
	other	10	13.2

From 120 respondents, 44 were interviewed at milk-selling points about their hygienic practices in milk handling. Among these, 88.6% said they used alkaline cleaners as the cleaning agent for milking equipment, and 11.4% used only water to clean their milk containers. In terms of storage, 79.6% of the respondents used only sealed containers to store milk for a certain period, while 20.5% made use of refrigerators in storing milk. On observation, it was noted that 70.5% of the milk-selling environment was in good hygienic condition, and 29.6% in poor condition.

Table 10. Practice of respondents related to milk handling at milk selling point

Condition considered	**Variable**	**Frequency**	**%**
What kind of cleaner do you apply to milking equipment?	Water only	7	11.4
	Soap and water	37	88.6
How do you store milk	Sealed container	35	79.6
	In refrigerator	9	20.5
Environmental condition of selling area	Good	31	70.5
	Poor	13	29.6

From 120 respondents, only 4.8% had some knowledge of *Campylobacter*. and its implications on milk safety when respondents were asked about their awareness of the organism, but 96.6% had no knowledge about the organism. From the questionnaire survey, 5% said they received training on hygienic milk handling practices, and 95% did not get the training. 5% of the respondents have been heard about milk-borne infection before, while 95% haven't heard about milk borne before, and all of the respondents believe in proper cleaning of the milking area reduce the risk of *Campylobacter* contamination.

Table 11. Knowledge of respondents about *Campylobacter* and milk contamination risks

Condition considered	**Variable**	**Frequency**	**%**
How much do you know about *Campylobacter* and the dangers it poses to the safety of milk?	Very familiar	0	0
	Somewhat familiar	0	0
	Not familiar	120	100
Have you received any training on preventing milk contamination?	Yes	6	5
	No	114	95
Have you heard milk borne infection	Yes	6	5
	No	114	95
Can proper cleaning of the milking area reduce the risk of *Campylobacter* contamination	Yes	120	100
	No	0	0
Have you heard milk borne infection	Yes	6	5
	No	114	95
Have you heard/know *Campylobacter*	Yes	0	0
	No	120	100

Out of 120 respondents, 93.4% think the importance of protective clothing during milking is to ensure milk safety, while 6.6% don't think it is as important. Furthermore, all respondents think that maintaining cleanliness in the milking area is essential to prevent contamination. The majority,

around 80.8%, consumed milk after boiling it, while 19.2% consumed it raw, and all respondents believe improper handling of milk could pose health hazards to consumers.

Table 12.Attitude of the respondents on *Campylobacter* and hygienic practice

Condition considered	**Variable**	**Frequency**	**%**
How important do you think the use of protective clothing (e.g., gloves, aprons) is during milking to ensure milk safety?	Important	112	93.3
	Not important	8	6.7
Do you believe maintaining cleanliness in the milking area is essential to prevent contamination?	Yes	120	100
	No	0	0
How did you and your family consume milk	After boiling	97	80.8
	Raw	23	19.2
Do you believe improper handling of milk could pose health hazards to consumers?	Yes	120	100
	No	0	0

5. DISCUSSION

Human *Campylobacter* gastroenteritis is mostly due to the consumption of animal-derived contaminated foods. According to Humphrey *et al.,* (2014) *Campylobacter* species colonize a wide range of intestines of wild and domestic animals inclusive of humans. The generally infecting organisms in humans are ingested through animal contaminated or undercooked products, including meat, milk, and dairy products. Other major sources include contaminated drinking water, direct contact with animals, faeces runoff from domestic animals, particularly chicken, and contamination of surface water (Asuming-Bediako *et al.,* 2019). The aim of the study was on assessment of milk handling practices and occurrence of *Campylobacter* in raw cow milk and associated environmental Sample along the milk supply chain at Maya city

The present finding indicated that the prevalence of *Campylobacter* occurrence in raw cow milk from different farms, dairy cows of local farmers, and milk selling points was 11.02%. This prevalence was significantly associated with factors such as poor cleanness of the house, use of unclean containers, and poor cow cleanliness. This prevalence rate of 11.02% is slightly higher but still within the range of the 9.1% reported in the Oromia Region, Ethiopia, by Eshetu et al. (2023), demonstrating a consistent presence of Campylobacter in these areas. It is also close to the 8.3% reported by Efimochkina *et al.* (2019) in Russia, However, the current study rate is considerably higher than the (1.3%) reported in the Jimma Zone, Southwest Ethiopia by, Gume *et al.,* (2023) but lower than the (18%) reported in Beni-Suef Governorate, Egypt, by Zeinhom *et al.,* (2021). These results are relatively lower than the (16%) reported by Admasie *et al.,* (2023), from major milk sheds in Ethiopia, as well as the 24.6% reported by (El-Zamkan and Hameed, 2016) in raw milk and other dairy products in Egypt, the (19.05%) reported by Ahmadi *et al.,* (2023) in Qazvin, Iran and also slightly less than (11.8%) reported by Andrzejewska *et al*., (2019) in Northern Poland and much lower than (37.11%)study conducted in the Eastern Cape Province, South Africa by reported (Igwaran and Okoh, 2020).

The discrepancies in prevalence rates may be attributed to variations in study design, sampling methods, and detection techniques. Factors such as sample size, seasonal variations, and differences in the timing of sample collection could also contribute to these differences. Additionally, variations in farm management practices, hygienic conditions, may further influence

the reported prevalence. Regional differences in climate, biosecurity measures, and milk handling practices can also play a role in these variations.", under the assumption is in line with Admasie *et al.,* (2024).

The findings from this study showed significant differences in the prevalence of *Campylobacter* between milk samples collected directly from the udders and those from farm fresh milk sold at selling points. The prevalence was significantly higher in farm fresh milk (bulk) from milk selling point (17.7%) as compared to udder milk (6.6%), this is in agreement with previous findings from other countries(Salihu *et al.,* 2010);(Bianchini *et al*., 2014) that contamination may occur post milking and during handling, storage or transportation of milk. Moreover, the detection of *Campylobacter* in the environmental samples of surface swabs, milker's hand swabs, and container swabs showed contamination prevalence of 15.0%, 5.0%, and 5.0%, respectively, indicating that environmental factors are a major cause contributing to milk contamination.

The findings also indicated there could be a possibility of milk contamination as a result of poor hygienic practices evidenced by the presence of *Campylobacter* spp. on milker's hands and containers. This adds to the higher rate of contaminants found in farm fresh milk, which underlines handling practices post milking as one of the major factors. Taken as a whole, evidence suggests that environmental contamination is an important pathway for the transmission of *Campylobacter* to milk, pointing to the need for better biosecurity measures on farms and at selling points; the cleaning regimes should be more thorough for containers, surfaces, and equipment, including hand hygiene by milkers themselves. This could by and large reduce the amount of contaminants. Assumption is in line with the(Knipper *et al.,* 2022).

In this study, several risk factors were assessed at each Dairy cow's milk sample taken correlated with the prevalence of *Campylobacter* occurrence. Hygienic practices such as cow cleanliness and cleanness of the House (AOR (95%CI) =5.7(1.21-40.20) with P-Value of 0.05 and (AOR (95% CI) = 14.35 (0.86-42.9) with P-value of 0.03 respectively, were significantly associated with the occurrence of *Campylobacter*. Raw milk from cows with poor cleanliness were 5.7 times more likely to have *Campylobacter* compared milk from cleaner cows and milk collected from poorly cleaned house were about 14.35 times more likely to be contaminated with *Campylobacter* than milk from cleaner house. This finding is in line with the report of previous studies (Gilpin *et al.,*

2008);(Kashoma *et al.*, 2016).And also hygienic practices such as hygiene of container (AOR(95%CI);6.63(1.28-34.35) with P-value 0.02,were significantly associated with the occurrence of *Campylobacter* at milk selling point. Specifically, the odds of *Campylobacter* presence are 6.63 times higher in cases where poor container hygiene is observed, compared to better hygiene practices.

In this study, 76 out of 120 respondents conducted a survey about their farm's and individual dairy cow holder's milk handling hygiene habits. Of them, 80.3% used water to clean the udders and teats prior to milking, and 19.7% used water mixed with antiseptic wipes. Although 9.2% of the individuals washed their hands only before and after milking, 90.7% of the respondents washed their hands after every step. Health status of cows was being checked by half of the responders daily and 34.2% once a week. Only 10.5% of the milk was stored under refrigeration after milking; the remaining 89.5% were stored at room temperature. A large majority (79%) of the milking facilities were in good condition, and for most (54%) of the milk transports, this was done within a few hours after milking. Only 5% of the interviewees received training on hygiene procedures. These findings emphasize the need for better training.

There are significant variations in hygiene measures according to the responses from respondents at milk-selling sites. For cleaning milking equipment, the majority (88.6%) utilized alkaline cleansers, while a minority (11.4%) only used water. The information about storage procedures revealed that 79.6% of the milk was stored in sealed containers, while only 20.5% used refrigeration, suggesting a possibility of milk spoiling. Although all the respondents were aware of the health implications of poor handling of milk, only 5.9% had any knowledge of *Campylobacter*. This reflects a significant level of unawareness of the disease. Based on observation, 70.5% of milk-selling areas were in good hygienic state, while 29.6% were in a poor hygienic state, indicating that some needed cleaning.

One of the limitations of the study was not using molecular techniques, such as PCR-based methods, for confirmation and differentiation of *Campylobacter* species. Conventional culture method was used; this may have poor sensitivity and specificity, leading to underestimation of true prevalence of *Campylobacter*. Molecular methods would have provided more detailed information on species identification and could also have detected strains that were hard to culture. And also

the inability to establish comparisons with past trends is mainly because of the deficiency of historical data concerning *Campylobacter* in the study area.

6.CONCLUSION AND RECOMMENDATIONS

The current study demonstrated that there is a significant prevalence of *Campylobacter* in fresh farm milk from a milk selling point, as well as udder milk from farms and individual dairy cows. The 11.0% prevalence rate of *Campylobacter* in raw cow milk does point to possible risks that *Campylobacter* poses to public health, particularly from contaminated milk in the supply chain. Risk factors such as house cleanliness, the overall hygiene of the milking environment and Hygiene of Milk Containers were found to be statistically significantly related to the prevalence of *Campylobacter*. These findings emphasize the necessity of improved hygiene practices throughout in the milk supply chain in study area. The strict procedure for cleaning and proper refrigeration of milk to impede the growth of bacteria is very essential in controlling the spread of *Campylobacter* and other foodborne pathogens. Moreover, increasing awareness among farmers, processors, and consumers about food safety is helpful in preventing the spread of *Campylobacter* and other foodborne pathogens.

Based on the above Conclusion, the following recommendations were forwarded:

- ✓ Strict hygiene procedures like routine cleaning and disinfection of milking sites, equipment, and containers by dairy cow farm workers, cow owners, and milk sellers should be implemented
- ✓ Educate farmers, milk sellers, and consumers about the potential risks of *Campylobacter* and the importance of proper milk handling and storage.
- ✓ Biosecurity on dairy farms should focus on improvement in animal, milking, and environmental hygiene.
- ✓ Future studies should incorporate molecular techniques, such as PCR-based methods, to improve the accuracy of *Campylobacter* detection and species differentiation
- ✓ Efforts should be made to establish baseline data for *Campylobacter* prevalence in the study area to enable trend analysis and better epidemiological understanding.

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9.APPENDIX

ANNEX I. Structured questionnaire for assessing milk hygiene and risk factors**.**

Respondents. farmers, dairy farm workers and milk sellers

Good morning/Good afternoon!

I would like to ask you questions concerning milk production procedures as well as your knowledge, attitude, and practices about the safety of milk and milk products. I am a researcher from the College of Veterinary Medicine at Haramaya University. The main topics of discussion will include your respondent's socio-demographic traits, farm management techniques, awareness of milk safety, and community customs and attitudes. We sincerely hope you would take the time to complete this survey, as the data we will be gathering will be extremely important for our future research on the hygienic practices involved in the production and processing of milk. Every inquiry is intended to gauge your thoughts and the current circumstances. We guarantee the privacy of your response and the non-sharing of any information gathered about you identify and the identification of your household with outside parties. We will get milk. samples from your cows and milk containers in addition to asking the questions so that any possible microorganisms in the samples can be examined in a lab. Nobody will learn your identity or the identity of your farm until the report for this study is prepared. It is completely voluntary to participate in this survey. There is no financial incentive to do so, and there won't be any obvious negative effects on you or your farm. You have the option to skip all of the questions. But as previously mentioned, it is crucial for our research that you Participate in this survey and respond to all of the questions.

Respondent statement:

I have understood above statements:

☐ Agree to participate on a voluntary basis.

☐ Not agree to participate.

(For interviewer)

If no agreement, pass to the next respondent.

Name of data collector _____signature _____

For further information:

Dr.Ishetu Namomsa Tel:+251-923-012795 E-mail:eshe2566@gmail.com

A. For farm worker/owner and management

1. Name of the farm worker _____

Address: district _____ Kebele _____village_____

2. Educational Status A. Illiterate B. Literate,

If the answer is "B", state the level_____

3. How do you ensure the cows' udders are clean before milking? A) Washing with Water. B) Using Disinfectant Wipes.C) Not cleaning

4. How often do you wash your hands during the milking process? A) After every use. B) Before and after.

5. How often do you check the health status of your cows? A). Daily B). Weekly C). When There Are Signs of Illness

6. Have you received any training on preventing milk contamination? A) Yes B) No

7. How is milk stored after collection? A) Refrigeration B) Room temperature

8.How quickly is milk transported from the farm to the selling point? A) Immediately B) Within few hours C) By the end of the day

9. Animal Housing and Cleanliness A) Good B) Poor

10. Type of material used to handle milk A) Aluminum B) Plastic C) other

B. Information and observation on hygienic practice of milk handling at milk selling point and from milk consumers

11. What kinds of cleaner do you apply to milking equipment A) Washing with Water

B) Soap and water

12. How do you store milk A) Sealed container B) In refrigerator

13. How much do you know about *Campylobacter* and the dangers it poses to the safety of milk?

A) Very familiar B) Somewhat familiar C) Not familiar

14. Do you believe improper handling of milk could pose health hazards to consumers?

A) Yes B) No

C) Information considered on milk borne infection

15. Do you like to consume milk

A Yes B No

16.Did you faced any health problem after consumption of raw milk

A) YES B) NO

17. How did you and your family consume milk

    A) After boiling B) Raw

18. Have you heard milk borne infection
19. Have you heard/know *Campylobacter*

    A) Yes B) No

ANNEX II. Observational for hygiene and biosecurity practices checklist and criteria

Table 1. Observational checklist for hygiene and biosecurity practices

Category	Check list item	Description	Remark
Hygiene Practices During Milking	Cow cleanliness	Cleanliness of the cow's udder and body before milking.	
	Milking Equipment	Equipment (milking machine, containers, utensils) are clean and sanitized before use.	
	Milker's Hygiene	Milker wears gloves and has clean hands before milking.	
Biosecurity Measures	Access Control	Visitors/workers follow biosecurity protocols (protective clothing, disinfect footwear	
	Sanitation Facilities	Availability of handwashing stations and sanitizers before and after milking.	
	Animal Health Monitoring	Proper health monitoring practices (e.g., quarantine of sick animals, preventive treatments).	
Milk Handling and Storage	Milk Temperature	Milk stored immediately after milking at the correct temperature (≤4°C).	
	Storage Equipment	Milk stored in clean, appropriate containers, properly sealed.	
	Handling and Transport	Milk is transported in clean, covered containers to avoid contamination.	
Farm Cleanliness and Environment	Milking Area Cleanliness	Milking area free from animal waste, dust, or other contaminants.	

	Animal Housing	Clean and well-ventilated animal housing.	
	Waste Disposal	Manure, bedding, and waste should not contaminate areas of milk storage or milking	
Milk Selling Point Hygiene	Milk Storage and Display	Milk: Stored in clean, covered containers, kept at proper temperature and free of contamination .	
	Selling Environment	Cleanliness and organization of the selling point (e.g., absence of waste, proper milk handling).	
	Dispensing Utensils	Milk dispensed with clean, sanitized utensils	
	Seller Hygiene	Seller puts on gloves or sanitizes hands before serving the milk; follows personal hygiene practices .	

CRITERIA TO CATEGORIZE AS GOOD OR POOR AND CLEAN OR DIRTY.

A) Udder Cleanliness: Clean Vs Dirty

- ✓ **Cean Udder;** The udder was considered "clean" if it did not have any visible dirt, manure or dried mud and was dry at the time of observation. Regular washing/wiping practices before milking were followed

- ✓ **Dirty Udder**; The udder was considered "dirty" if at the time of observation, it had visible dirt, manure, or dried mud, or was wet, sticky, or contaminated with any kind of debris.

B) Milking Area Hygiene: Good Vs. Poor

- ✓ **Good Hygiene;** The milking area was considered "good" if the floor was clean and dry, with no accumulation of manure or bad odor, proper drainage, and cleanliness practices could be seen, such as regular cleaning or disinfection.
- ✓ **Poor Hygiene;** A milking area was considered as "poor" even with a dirty or wet floor with dung accumulation, having a strong foul odour, poor drainage, and no signs of regular washing.

C) Hygiene of Milker: Good Vs. Poor

- ✓ **Good Hygiene;** Milkers were categorized as having "good" hygiene if they wore clean clothing, practiced handwashing before milking, and avoided using dirty hands or materials during milking.
- ✓ **Poor Hygiene;** Milkers were considered to have "poor" hygiene if they wore visibly dirty clothing, did not wash hands before milking, or used unclean materials, such as contaminated towels or milking equipment.

D) Cow Cleanliness: Clean vs Dirty

- ✓ **Clean cow;** A cow was considered "clean" if its body, especially the flanks, legs, and udder region, showed no apparent dirt, manure, or mud. The cow would be considered clean if its coat appeared dry and well-kept, indicating regular grooming and good management.
- ✓ **Dirty Cow;** A cow was considered "dirty" if its body showed visible filth, dung, or other foreign materials caked thereon. Spilled or splattered wet or sticky spots on

the coat caused by improper care or improper environment also constituted "dirty," too.

E) Cleanliness of Selling Environment; Good vs. Poor

- ✓ **Good Environment;** The selling environment was considered "good" if the milk was in clean containers, free of flies/insects, the temperature of the milk was appropriate, and there was no source of contamination seen in the nearby area.
- ✓ **Poor Environment;** The milk selling environment was considered "poor" if milk was stored in dirty or uncovered containers, exposed to flies or insects, or in a visibly unclean and unhygienic environment.

D) Animal Health Status.

- ➢ **Good Health Status**:

Animals are considered to have a good health status when they exhibit the following

- ✓ No observable clinical signs of illness (e.g., coughing, diarrhea, nasal discharge)
- ✓ Clear, bright eyes and no excessive tearing or redness
- ✓ Normal appetite and feeding behavior.
- ✓ Smooth, shiny coat with no bald patches, sores, or skin infections
- ✓ Proper body condition score (BCS) based on species-specific standards

- ➢ **Poor Health Status**

  - ✓ Visible signs of illness, such as coughing, diarrhea, nasal discharge, or lethargy
  - ✓ Dull, sunken eyes or excessive tearing
  - ✓ Loss of appetite or reduced feeding behavior
  - ✓ Poor body condition score (e.g., emaciated or overly thin animals)

ANNEX III. Data collection format

Date _____

Name of the farm _____size of the farm_____site/location_____

Kebele_____ total animal in the farm_____

Table 2. Record sheet used during sample collection and processing

Serial Number	Sample Source	Farm Code	Date of Collection	Date of Culturing On MCCDA	Growth on Mccda	Colony Characteristic On Mccda	Gram Staining	Catalase Test	Oxidase Test	Result

Procedure to collecting milk sample:

ANNEX IIIV. Media preparation protocols according to manufacturer instructions

Bolton Broth Enrichement preparation method

1. Dissolve the broth base in distilled water
2. Sterilize by autoclaving at 121°C for 15 minutes.
3. After cooling to around 45-50°C**,** add the antibiotic supplements aseptically. (Bolton supplement).
4. Add 50ml Defibrinated horse blood (enhances *Campylobacter* growth).
5. Mix thoroughly and store at 2-8°C.

***Campylobacter* Blood-Free Selective Agar (CCDA)**

Campylobacter Blood-Free Selective Medium (CCDA) is one of several media formulations available for the selective isolation of *Campylobacter* species. Primarily C. *jejuni* and C. *coli*. CCDA was described by Bolton. And formulated to replace blood with a combination of charcoal, ferrous sulphate, and sodium pyruvate. CDA is recommended for food testing. CCDA with the addition of yeast extract and cefoperazone is used in the isolation of *Campylobacter species*. From foodstuffs and swabs in the FDA/BAM method. This product complies with the requirements of ISO 10272-1:2006.

Preparation.

Suspend 45.5g of the medium in one liter of deionized / purified water. Allow the medium to soak whilst mixing for 10 minutes. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Sterilise at 121°C for 15 minutes. Cool to 45-50°C, add 2 vials of *Campylobacter* (Preston) supplement (LS0010), mix well and aseptically dispense into appropriate sterile containers**.**

ANNEX IV. Laboratory procedure

1 Gram's Staining Procedure

1 Make a thin bacterial colony smear and allow it to dry on the air

2. Fix the dried smear by passing through the Bunsen flame two to three times taking care not to overheat the smear

3 Flood the fixed smear with Gram's crystal violet (primary stain). Let stand for 60 seconds.

4 Pour off the stain and gently wash with tape water.

5 Flood with Gram's iodine solution and gently wash with tape water

6 Pour off the iodine solution and gently wash with tape water.

7 Decolorize with Gram's decolorizer solution 95% acetone alcohol) for 15-20 seconds

8 Counterstain with Gram's safranin solution or carbolfuchsin (counter stain) for 60 seconds

9 Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shake to remove most of the water and air dried.

10 Examine the finished slide under a microscope (oil immersion objective)

11 Interpretation: bluish purple color indicate Gram positive and pinkish color indicate gram negative bacteria.

2. Catalase Test Procedure

1 Pick a colony from a culture and place it on a clean glass slide.

2 Put one drop of 3% H2O2 over the organism on the slide

3 Observation for immediate bubbling (gas liberation) and record the result

4 Interpretation: A positive result is the rapid evolution of O_2 evidenced by bubbling and a negative result is no bubbles or only few scattered bubbles.

3. Oxidase Test Procedure

The method is based on the principle that certain phenylene-diamantes-derivatives are Oxidized by cytochrome C to produce a bluish indophenol. Commercial kits are available.

Step; 1 Transfer one colony to a filter paper.

2 Soak the filter in an oxidase solution.

Interpretation: Appearance of a blue color within 10 sec indicates a positive result

Figure 1. Some figures captured during the study period

During milk sample collection

Preparation of Bolton broth enrichment media during lab. Procedure