ENUMERATION AND DETECTION OF ANTIBIOTIC SUSCEPTIBILITY PATTERN OF COLIFORM BACTERIA FROM MILK SAMPLES IN KATHMANDU.

A PROJECT WORK SUBMITTED TO THE

DEPARTMENT OF MICROBIOLOGY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

BACHELOR OF SCIENCE (B.Sc.) IN MICROBIOLOGY

BY

SURAJ CHAULAGAIN

SYMBOL No., 500330160

T.U. REGISTRATION No., 5-2-33-164-2017

JUNE, 2022

Tribhuvan University
Institute of Science & Technology
AMRIT CAMPUS
P. O. Box No. 102, Thamel, Kathmandu, Nepal.
E-mail : amritcampus@ntc.net.np
☎ 4411637
4410408

Amrit Campus

Ref No.:

Date:

RECOMMENDATION

This is to recommend that **Suraj Chaulagain,** Symbol number 500330160, TU Registration number 5-2-33-164-2017, has carried out project work entitled as" "**Enumeration and Antibiotic Susceptibility Pattern of Coliform Bacteria from Milk Samples in Kathmandu"** for the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology under my/our supervision in Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

To my/ our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the submission of the project work for the partial fulfillment of Bachelor of Science (B.Sc.) degree.

.....

Asst. Prof. Suchitra Thapa

Supervisor

Department of Microbiology

Amrit Campus

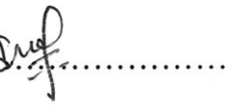
Tribhuvan University (T.U.)

15, JUNE, 2022

DECLARATION

This project work entitled as **"Enumeration and Antibiotic Susceptibility Pattern of Coliform Bacteria from Milk Samples in Kathmandu"** is being submitted to the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the partial fulfillment of the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology. This project work is carried out by me under the supervision of Suchitra Thapa in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

This work is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.



Suraj Chaulagain

Symbol No. 500330160

T.U. Registration No.5-2-33-164-2017

19 June, 2022

Tribhuvan University
Institute of Science & Technology
AMRIT CAMPUS
P. O. Box No. 102, Thamel, Kathmandu, Nepal.
E-mail : amritcampus@ntc.net.np
4411637
4410408

Ref No.:

Date:

LETTER OF FORWARD

20, June, 2022

On the recommendation of **Suchitra Thapa,** this project work is submitted by **Suraj Chaulagain**, Symbol No. 500330160, Registration No. 5-2-33-160-2017, entitled as **"Enumeration and Antibiotic Susceptibility Pattern of Coliform Bacteria from Milk in Kathmandu"** is forwarded by the Department of Microbiology, Amrit Campus, for the approval to the Evaluation of Committee, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

He has fulfilled all the requirement laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the project work.

.....

Asst. Prof. Suchitra Thapa

Head of Department

Amrit Campus

Tribhuvan University

Tribhuvan University
Institute of Science & Technology
AMRIT CAMPUS
P. O. Box No. 102, Thamel, Kathmandu, Nepal.
E-mail : amritcampus@ntc.net.np
4411637
4410408

Ref No.:

Date:

BOARD OF EXAMINATION AND

CERTIFICATE OF APPROVAL

This project work (PRO-406) entitled as **"Enumeration and Antibiotic Susceptibility Pattern of Coliform Bacteria from Milk Samples in Kathmandu"** by Suraj Chaulagain, Symbol No. 500330160, Registration No. 5-2-33-164-2017 under the supervision of Suchitra Thapa in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), is hereby submitted for the partial fulfillment of Bachelor of Science (B.Sc.) degree in Microbiology. This report has been accepted and forwarded to the Controller of Examination, Institute of Science and Technology, Tribhuvan University, Nepal for the legal procedure.

.....

Asst. Prof. Suchitra Thapa
Supervisor
Department of Microbiology
Amrit Campus
Tribhuvan University

.....

Asst. Prof. Suchitra Thapa
Head of Department
Amrit Campus
Tribhuvan University

.....

Assoc. Prof. Dr. Dev Raj Joshi
External Examiner
Central Department of Microbiology
Tribhuvan University
Kritipur

.....

Asst. Prof. Munal Subedi
Internal Examiner
Department of Microbiology
Amrit Campus
Tribhuvan University

27 June, 2022

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.....

Suraj Chaulagain

Symbol No. 500330160

T.U. Registration No. 5-2-33-164-2017

June, 2022

ABSTRACT

The presence coliforms and their resistance in milk is the big issue in present time. Milk is an excellent source of nutrients and also serves as a good medium for the growth of milk-borne pathogens. Cross-sectional study was conducted to assess and compare microbial quality of raw milk and pasteurized milk and also determine antimicrobial susceptibility patterns of coliforms from the milk samples. For this, 30 milk samples (15 raw and 15 pasteurized milk) were collected from different locations of Kathmandu district. Starch adulteration test and MBRT were done. TCC, FCC for each sample were determined by pour plate technique and interpretated with BIS guidelines (1992), DFTQC guidelines and identification was done. Antibiotic susceptibility testing of isolates was carried out by Kirby Bauer disk diffusion method using 12 different antibiotics. TCC of the 12 raw samples were higher than the guideline and its FCC was also found to be higher in 9 samples. In case of pasteurized samples, TCC was higher in 6 samples and FCC in 4 samples. A total of 31 isolates, 21 from raw samples and rest from pasteurized samples were identified. Out of 31 isolates, 17 (54.84%) were identified as *Klebsiella* spp., 13 (41.94%) were *E. coli* and 1 (3.22%) was *Citrobacter* spp. AST of coliform isolates were 100% sensitive against TE. 96.77% of the isolates were sensitive towards NIT, PIT, COT, C and AK. Out of total, 11 (35.48%) were MDR (Multi-Drug Resistant). Among them, 7 (63.64%) were from raw samples and rest 4 (36.36%) from pasteurized samples. Although, quality of most of the samples were good as per MBRT but the presence of antimicrobial resistant bacteria and adulterants questions the overall quality of milk. Thus, it is concluded that the milk produced by small-scale farm from the studied area are not of good quality, caused by coliforms especially the antibiotic resistant. Therefore, such type of study for monitoring the microbial quality of milk should be done in order to safeguard the consumers. Otherwise, it will be hazardous for the consumers and can be a potential source of milk-borne infections.

Key words: Antibiotic susceptibility testing, Multi drug resistant, *E. coli*, Total coliform count (TCC), Fecal coliform count (FCC).

शोधसार

दुध पौष्टिक तत्वको राम्रो स्रोत हो र यसमा विभिन्न किसिमको ब्याक्टेरियाहरु बाच्न सक्छन्। गुणस्तर रहित दुधको सेवनबाट विभिन्न प्रकारका रोगहरु लाग्छ ।स्वास्थको दृष्टिकोणले दुधको गुणस्तर मापन भएको दुधको मात्र सेवन गर्नुपर्छ । यस अध्ययनमा दुधको गुणस्तर मापनका लागी विभिन्न विधिहरु अपनाइयो । यस अध्ययनमा दुधको, total coliform count, fecal coliform count आदि जाँच गरिएको थियो । सोडा तथा स्टार्चको मिसावट, MBRT र Antibiotics susceptibility test गरिएको थियो । AST का लागी Kirby Bauer Disc diffusion method अपनाइएको थियो । यस अध्ययनमा ३० वटा दुधका नमुनाहरु सङ्कलन गरिएको थियो । जस अन्तर्गत १५ ओटा ताजा दुध र १५ वटा पास्चराइज दुधका नमुनाहरु काठ्माण्डौंको विभिन्न भागबाट सङ्कलन गरियो । यस अध्ययनमा १७ वटा नमुनाहरुमा सोडाको मिसावट भएको पाइयो र स्टार्चको मिसावट भने पाइएन । यस अध्ययनमा दुधको Total coliform count, Fecal coliform count, MBRT, Adulteration आदि जाँच गरिएको थियो । TCC र FCC का लागि Pour plate method अपनाइएको थियो । जसका लागि Petri-plates लाई २ फरक तापक्रम (३७°C र ४४°C) मा २४ घण्टासम्म इन्कुवेट गरिएको थियो । त्यस पश्चात्, उक्त plates मा भएका कोलिफम गनियो र नतिजालाई BIS र DFTQC Guideline सँग तुलना गरियो । उक्त plates मा भएका isolates लाई inoculating loop को मद्धतले nutrient broth मा राखियो र ४ घण्टा पछि त्यसलाई MacConkey Agar मा Sub-Culture गरियो र Biochemical Tests को आधारमा त्यसको Identification गरियो । पत्ता लगाइएको Isolates को Antibiotic Susceptibility Pattern जाँच गरियो ।

Total coliform count गर्दा दुवै दुधमा गरि ६०% र Fecal coliform count भने ४३% दुधमा पाइयो । Antibiotics Susceptibility Test मा सबै भन्दा धेरै resistant drug मा AMP, CX, NA र CIP घट्दो क्रममा परेका थिए । जम्मा ११ ओटा एम्.डी.आर. ब्याक्टेरिया पनि भेटिएको थियो । यस अध्ययनबाट हामीले काठ्माण्डौंमा वितरण भइरहेको दुधको गुणस्तर थाहा पाउन सक्छौं ।

मूख्य शब्दहरु: एम.बि.आर.टी, ए.एम्.पी., सी.एक्स्, एन्.ए.,सी.आइ.पी, डि.एफ्.टि.क्यु.सी, माइक्रोवियल क्वालिटी

LIST OF ACRONYMS AND ABBREVIATIONS

AST	Antibiotic Susceptibility Testing
BIS	Bureau of Indian Standard
CFU	Colony Forming Unit
CLSI	Clinical Laboratory and Standard Institute
DFTQC	Department of Food Technology and Quality Control
FCC	Fecal Coliform Count
MBRT	Methylene Blue Reduction Test
MDR	Multi-Drug Resistant
MHA	Muller Hinton Agar
NB	Nutrient Broth
VRBA	Violet Red Bile Agar
TCC	Total Coliform count

LIST OF SYMBOLS

µm	Micro-meter
+	Positive
-	Negative
˚C	Celsius
%	Percentage

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CHAPTER 1

1. INTRODUCTION

1.1 General Introduction

Milk, with its high nutritional value, around 87.80% water, 3.20% proteins, 3.50% fat, 4.80% lactose, & 0.70% mineral content is considered to be 'complete meal'. It also serves as rich source of energy, 100gram milk providing around 66 Kcal of energy (Pehrsson,2000). High nutritive value of milk also makes it an ideal medium for the rapid multiplication of bacteria, particularly under unhygienic production and storage at ambient temperature.

Most of the people in the world consume pasteurized milk and few people prefer raw milk as they believe that raw milk is more beneficial, tastier and convenient than pasteurized one (Altalhi and Hassan 2009). Generally, microbial contamination in milk can be minimized through adherence to effective good hygienic practices at farm level, and in order to protect the public milk-borne infection, it is important to screen milk which is informally taken to the market. Also, can be achieved through proper boiling or pasteurization of raw milk before processing and consumption. The lack of awareness of milk-borne infections in many developing countries and consumption of raw milk predispose small-scale livestock keepers, consumers and the general public at risk of contracting these infections (Mosalagae *et al* 2011). Adulteration means addition of substances other than the normal constituents of milk. Starch, sugar, soda, etc., are the substances normally adulterated in milk. Besides, addition of water is also common practice among the farmers. Such adulteration lowers the quality of milk and harms the consumers. So, such practice must be discouraged. Starch is not a normal constituent of milk. If added, it increases the Solid not fat of the milk. So, milk can be diluted and starch is added which screens the dilution. In such milk, actual nutrient is low. The adulteration of starch in milk can be detected by performing iodine test. Similarly, soda, table sugar and other adulterants is not a good act, as it may cause life threatening disease to the consumer. So, such act should be discouraged and strict rules should be made (Manandhar, S., (2013)).

Sometimes, dairy animals serve as major reservoirs for many milk-borne pathogens such as *Staphylococcus aureus* and *Escherichia coli* (*E. coli* O157:H7). A number of milk-

borne epidemics and outbreaks, such as tuberculosis, typhoid, diphtheria, dysentery, etc., have been occurred through consumption of milk and their product in humans (khan *et al*.,2018). These microbes may gain entry into raw milk in numerous way such as directly from dairy buffaloes experiencing subclinical or clinical mastitis, contaminated water source used for washing and utensils used for the storage on farm, during transportation. Pasteurization helps to minimize all these possible cases. Animals affected with mastitis might shed large numbers of microorganisms into the milk. Many milk-borne epidemics of human diseases, that occur due to contamination of milk by inappropriate handling by dairy workers, unclean utensils; non-potable water used as adulterants (Chatterjee *et al*.,2006). To minimize possible health hazard by the consumption of raw milk, pasteurization can be done.

An analysis of milk-borne outbreaks of infectious intestinal disease in England and Wales from 19922000 identified unpasteurized milk as the most common vehicle (52% of the milk-borne outbreaks), and pasteurized milk as the second most frequent (37%). Of the outbreaks attributed to pasteurized milk, inadequate heat treatment was the most common fault responsible, followed by cross contamination and inappropriate storage (Gillespie *et al* 2003). Coliform contamination ranks high among the most common types of contamination in the dairy industry. Microorganisms such as *E. coli, Pseudomonas aeruginosa*, *Citrobacter* spp., *Klebsiella* spp. and *Proteus mirabilis* can multiply in the normal summer temperatures and hence unpasteurized milk has every chance of containing *E. coli*. Therefore, even nowadays, basic microbiology tests performed on milk or any dairy product are aimed at detecting coliforms (Nellutla, A *et al.* (2012)).

In recent years, *Escherichia coli* has become recognized as a serious food borne pathogen and has been associated with numerous outbreaks of disease in the UK, Japan, and USA (Uyttendaele *et al* 1999; Scotter *et al* 2000). Recovery of E. coli from food is an indicative of possible presence of entero-pathogenic and/or toxigenic micro-organisms which could constitute a public health hazard. *Enteropathogenic E. coli* (EPEC) can cause severe diarrhea and vomiting in infants and young children (Sousa 2005).

E. coli strains that are resistant to various antibiotics are of particular concern for global health so they are common entero-pathogenic bacteria. It has been confirmed that approximately 7% of E. coli isolates identified in raw milk are multi-drug resistant (Rasheed *et al* 2014).

To minimize possible health hazard by the consumption of raw milk, pasteurization can be employed. Pasteurization kills any pathogens that might be present in milk sample (especially *Mycobacterium tuberculosis*, *Salmonella* spp., enteropathogenic *E. coli*, *Campylobacter jejuni*, and *Listeria monocytogenes*). Most of the spoilage microorganisms in raw milk, such as coliforms, mesophilic lactic acid bacteria, and psychrotrophs can deteriorate products and may play significant role in the aspects of consumers health as well.

1.2 Rationale

Milk and milk product can become microbiologically hazardous to consumer when the principles of hygiene and sanitations are not met. Sometimes, it is spoiled and become worthless due to the contamination by pathogens from humans or from environments during production, processing, and preparation or it may originate from animals. The random use of antimicrobial agents in animals is the reason behind the expansion of antimicrobial resistant bacteria that may be transferred to human through contact, contaminated environment or milk and milk products (Syit, 2008; Sharma *et al*., 2011). Such conditions may become vehicle for transmission of diseases. Thus, examination of quality of milk allows us to determine the existence of these hazards. Such hazards can be minimized by processing the sample properly by the process known as pasteurization. Other than this, the microbial contamination can be minimized by following the guidelines during collection, packaging and distribution. The antimicrobial resistant can be minimized by controlling the random use of antimicrobial agents. Thus, monitoring the quality of milk before consumption or processing is prerequisite.

1.3 Objectives

1.3.1 General objective

To enumerate and determine the antibiotic susceptibility pattern of coliform bacteria from milk samples in Kathmandu.

1.3.2 Specific objectives

1. To detect adulteration (i.e., adulteration of Starch, Soda) in milk samples.
2. To determine Total Coliform Count (TCC) and Faecal Coliform Count (FCC) of samples.
3. To isolate and identify coliforms.
4. To determine antibiotic susceptibility pattern of the coliforms.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Definition and composition of Milk

Milk is defined to be the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, five days after and 15 days before parturition, which contains not less than 8.5% solid not fat and not less than 3.5% milk fat (U.S. Public Health Services, 1965).

The microbial flora of raw milk consists of those organisms that may be present on the cow's udder and hide and on milking utensils or lines. Under proper handling and storage conditions, the predominant flora is gram positive. While yeasts, moulds, and gram- negative bacteria, may be found along with lactic acid bacteria. Most or all of these types are most sensitive than gram-positives and are more likely to be destroyed during pasteurization (Jay, James M.,2005). Pasteurized milk is obtained heating milk for a minimum time of 15 second (at temperature of 72^{o} C) or 30 minutes (at a temperature of 63^{o}C) (Pawar, J. and Mulye, Kalpita, 2021).

2.2 Types of milk and their importance

Commercially available milk can be classified into two major groups: liquid milk and dried or powdered milk. Milk is available with different fat content including whole (3.25%), reduced fat (2%), low-fat or light (0.5, 1%), non-fat/ fat-free/ skim (<0.5%) (Vaclavik and Christian 2007).

2.2.1 Raw milk

European parliament and the council of European Union 2004 defines raw milk as "the milk produced by the secretion of the mammary gland of farmed animals, which has not been heayed more than 40℃ or has not undergone any treatment with an equivalent effect". Most of the people in the world consume pasteurized milk and few people prefer raw milk as they believe that raw milk is more beneficial, tastier and convenient than pasteurized one (Altalhi and Hassan 2009).

2.2.2 Pasteurized milk

Pasteurization, a preservation technique for milk, is mainly performed to destroy or inactive all the harmful or pathogenic microorganisms by using heat treatment. Pasteurized milk is obtained by heating milk for minimum time of 15 seconds (at a temperature of 72℃) or 30 minutes (at a temperature of 63℃). Ultra-High Temperature (UHT) pasteurization is a process of heating milk at temperature of 135℃ - 150℃ for the fraction of second holding time to prolong the shelf life of milk (Pawar, J. 2021).

The use of milk products as human food has got a very long history. It contains in a balanced form of all the necessary and digestible elements for building and maintaining the human and animal body. Research has shown that milk products have an immune enhancing property as well, particularly for the benefit of HIV/AIDS affected people. In addition, milk contains various properties, which make it easy to convert into different milk products or to use it as an ingredient for other food items. Various human cultures have their own traditional ways of using milk and preparing different milk products (WHO 2003).

2.3 Microbial contamination and sources of milk

Milk obtained from healthy animal's udder is free from pathogenic bacteria but some of the animals in field condition may be suffering from sub-clinical mastitis and are excreting the causative agent in milk, such milk contaminates the bulk milk. Moreover, fresh milk may get microbial contamination from utensils, animal skin, environment, or water used for cleaning etc. (FAO 2008). Mastitis, external udder surfaces, inadequate cooling of the milk, improper udder preparation methods, unclean milking equipment and the water used for cleaning purposes are considered as the main source of milk contamination (Dehinenet *et al* 2013).

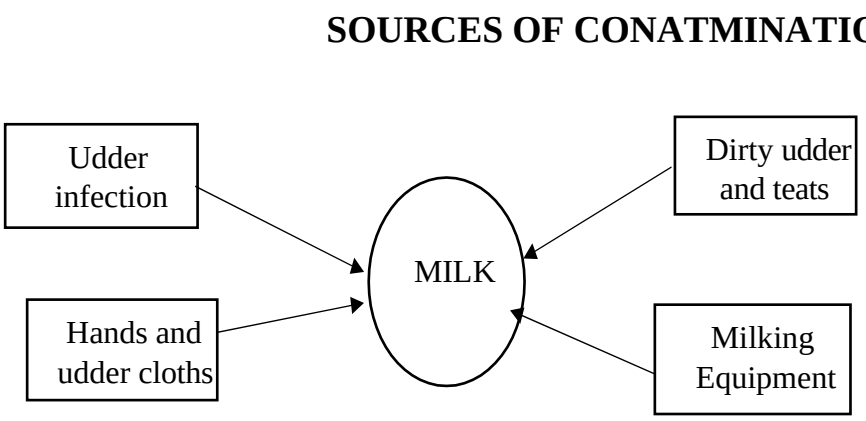


Figure 1 Major sources of contamination in milk (National Mastitis Council, 2005)

2.4 Milk-borne diseases and pathogenic microorganisms

Foodborne diseases are the common and widespread global problems. Several outbreaks have been reported as a result of consuming contaminated milk that may look, taste and smell perfectly normal but are in fact contaminated with large number of harmful bacteria (CDC 2009). Foodborne illnesses account for 48 million infection per year in the United States of America, with Norovirus, *Salmonella* spp. (Non-typhoidal), *Clostridium perfringens*, *Campylobacter* spp. and *Staphylococcus aureus* ranking as the top five pathogens contributing to domestically-acquired foodborne illnesses (CDC 2011). The diseases transmitted by milk include tuberculosis, typhoid fever, scarlet fever, septic sore throat, undulant fever, gastroenteritis, diphtheria (Manay *et al* 1987). Other diseases include dysentery, food poisoning, anthrax and para-typhoidal fever (Jensen *et al* 1989).

Table1: Bacterial disease due to contaminated milk Diseases

	Causative organisms
Cholera	*Vibrio cholera*
Gastroenteritis	*Enteric E. coli*
Typhoid fever	*Salmonella* Typhi
Paratyphoid fever	*Salmonella* Paratyphi
Salmonellosis fever	*Salmonella enteritidis*
Shigellosis	*Shigella sonnei*
Pneumonia	*Klebsiella pneumoniae*

(Source: Shrestha. P 2012 microbial study of milk)

2.5 Coliforms

Coliforms are defined as facultatively anaerobic, gram-negative, non-sporing, rod-shaped bacteria that ferment lactose with the production of acid and gas within 24 hours of incubation at 35℃ (Aneja, K.R., 2014).

2.5.1 Escherichia coli

Escherichia coli also known as *E. coli*, is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Tenaillon, O *et al* 2010). Although E. coli is a part of the normal flora of the intestinal tract, certain strains can cause a moderate to severe gastroenteritis in humans and animals. Enteropathogenic strains colonize the jejunum and upper ileum of the small intestine and cause acute gastroenteritis in new-borne and in infants up to 2 years of age. Entero-invasive strains invade the epithelial cells of the large intestine and cause diarrhoea in older children and adults. Enterotoxigenic (enterotoxin-producing) strains produce one or both of two different toxins: a heat stable toxin (ST) and a heat labile toxin (LT). Both toxins cause diarrhoea in adults and infants. The LT stimulates adenylate cyclase activity in a manner similar to that of cholera toxin, whereas the ST stimulates guanylate cyclase activity. Enterotoxigenic strains of E. coli are often associated with traveller's diarrhoea, a common disease contracted by tourists when visiting developing countries. Other strains of *E. coli* which are usually harmless in their normal habitat (the intestine) can cause disease when they gain access to other sites or tissues. These diseases include urinary tract infections, septic infections, bacteriaemia, meningitis, pulmonary infections, abscesses, and skin and wound infections (Pelczar, M 2015). Faecal bacteria (Thermotolerant coliforms) have been used as an indicator to the possible presence of pathogens in surface waters and the risk of disease based on epidemiological evidence of waterborne diseases. Consequently, because of the difficulties to detect the many possible

pathogens concentrations of faecal bacteria including thermotolerant coliforms, enterococci and *E. coli*, are used as the primary indicators of faecal contamination (Elayse, M., Hachich *et al.* 2012).

2.5.2 *Klebsiella* spp.

These are short plump (1-2 x 0.5-0.8µm) Gram-negative bacilli, non-motile and capsulated. They ferment sugars (glucose, lactose, sucrose, mannitol) with production of acid and gas and split urea by means of urease. They do not produce indole, are usually MR negative and VP and citrate positive (IMViC --++). They grow well in blood agar and MacConkey's agar producing large, pink and mucoid colonies (due to presence of capsule). In the current classification, the tribe Klebsiellae includes four major genera: *Klebsiella*, *Enterobacter, Hafnia,* and *Serratia*. In 1989, a new fifth genus, *Pantoea*, has been included in the *Klebsiella* tribe which was previously known as *Enterobacter agglomerans*, now called *Pantoea agglomerans.* The genus *Klebsiella* was named after Edwin Klebs, a late- 19th century German Microbiologist.

2.5.3 *Citrobacter* spp.

These motile, citrate positive bacilli are normal inhabitants of intestine. They grow in ordinary media, ferment lactose late, or not at all. The genus, *Citrobacter* contains eleven genomospecies – *C. Freundii*, *C. koseri*, and *C. amalonaticus* are of medical importance. Other species are not of medical importance and *include C. braakii, C. farmer, C. gillenii, C. murliniae, C. rodentium, C. sedlakii, C. werkmanii*, and *C. youngae*. All species of Citrobacter except *C. koseri* have been obtained predominantly from stools. Some strains of Citrobacter show antigenic sharing with salmonellae, e.g., a Vi antigen possessed by the Bhatnagar strain, which is serologically identical with the Vi antigen of *S. typhi* and *S. paratyphi C*. This may lead to confusion in laboratory diagnosis. Citrobacter can cause urinary infection and sepsis. *Citrobacter* are motile, utilize citrate, grow in KCN medium, and do not produce indole. *C. freundii* produces H_2S but *C. koseri* does not form H_2S (Chakraborty 2013).

2.5.4 Source to milk

E. coli, *Klebsiella* spp. and *Citrobacter* spp. are carried in the intestinal tract of ruminants, including domestic animals used in milk production, e.g., cows, sheep and goats. Contaminated food, especially undercooked ground beef, unpasteurized (raw) milk and juice, soft cheeses made from raw milk, and raw fruits and vegetables (such as sprouts) are also a source of *E. coli* contamination.

Raw milk can be a significant source of food-borne pathogens, and there have been numerous food-poisoning outbreaks associated with direct consumption of raw milk that has been inadequately heat-treated, or milk that has been re-contaminated after heat treatment. The presence of pathogens in milk is likely to arise from contamination by faecal material during the milking process. Contaminated milking equipment and floors can facilitate the spread of these pathogens to the udders; subsequently, milking equipment including teat cups, pipelines, filters and bulk storage vessels can become colonized (O' Loughlin and Upton 2001). Nowadays, faecal coliform contamination in milk can be generally seen due to sanitary contamination and poor hygiene practice in the farms which are the sources through which the entrance of faecal coliform is possible.

2.6 Prevention and control of microbial contamination in milk

Generally, microbial contamination in milk can be minimized through adherence to effective good hygienic practices at farm level; and in order to protect the public against milk-borne infections it is important to screen milk which is informally taken to the market. Prevention and control of microbial quality of milk is through elimination of organisms from human carriers by general improvements in water supplies, public health education, personal and environmental hygiene.

Also, can be achieved through proper boiling or pasteurization of raw milk before processing and consumption. Pathogenic organisms from the lactating animals can be controlled through improvements in animal husbandry and maintenance of good animal practices, and those from the environments and equipment can be prevented by adhering to general hygienic practices and environmental cleanliness (Mosalagae *et al* 2011).

The primary controls of the microbes in raw and processed milk are limiting the time and temperature of storage, ensuring any processing is performed effectively and paying close attention to equipment cleaning and sanitation. Secondary controls, such as the use of carbon dioxide, bacteriocins, LAB and antimicrobial proteins, are appropriate in certain circumstances. In the dairy industry, the ultimate control is by heat treatment. Some non-thermal treatments are also effective and may find commercial application in the future (Tamime 2009). Potential sources of contamination in milk are dung, water, utensils, soil, feed, air, milking equipment, animal and the first and most important steps in clean milk production. Clean milk production results in milk that is safe for human consumption, free from disease producing microorganisms, has a high keeping quality and high commercial value and high-quality base suitable for processing, resulting in high quality finished products (Kanyeka 2014).

2.7 Antibiotics and their resistance

Antimicrobial agents particularly antibiotics are veterinary drugs used in dairy cattle for treatment and prevention of various diseases. Also, they are used to improve feed efficiency, increase milk production or as growth promoters (Syit 2008; Sharma *et al* 2011). Antibiotic use sometimes occurs in response to several challenges that face the livestock industry that include high level of stress, diseases, poor animal genetic potential, poor management, poor nutrition and drought (Mellau *et al* 2010).

Commonly used antimicrobial agents particularly antibiotics in farm level are of different groups or classes. These include the penicillins, tetracyclines, aminoglycosides, beta-14 lactams, sulphonamides, macrolides, and phenicols (Bukuku 2013). These antibiotics may be used singly or sometimes in combination when treating cattle.

It is also important to recognize the types of germs which can be transmitted through insufficient thermal preparation of milk or milk products or through post-pasteurization contamination, in order to successfully avoid transmission of milk-borne infections (Dhanashekar *et al* 2012). In order to avoid these transmission proper milking, cleaning and sanitizing procedures of equipment and environments are essential (Kanyeka 2014).

2.7.1 Drug resistance in coliforms

Coliform bacteria develop significant resistant on administration of antibiotics. Several bacteria in coliform group such as *E. coli* isolated from 22 raw milk samples exhibited 100% resistant against Rifampin (R) and Tetracycline (TE) and 50% resistance against Nalidixic Acid (NA) but were 100% sensitive against Imipenem (IMP) (Uddin *et al* 2011). Out of 66 milk samples collected from Kathmandu valley, *E. coli* was isolated from 18.8% pasteurized, 40% of unpasteurized and 20% of the raw milk samples respectively. 16.7% (n=180) of *E. coli* isolates were susceptible to ampicillin whereas, 100% isolates were susceptible to other tested antibiotics (Acharya *et al* 2017). From 58 out of 70 raw cow milk samples which showed positive isolating result after morphological and biochemical tests, 38% (22) *E. coli* were isolated. The isolates showed highest resistivity towards Ampicillin (98%) and ciprofloxacin (91%) (Badri *et al* 2017).

2.7.2 MDR in Coliform

Coliform bacteria such as *E. coli*, *Klebsiella* spp., *Citrobacter* spp. etc., develop significant resistant towards three or more classes of drug. In this study, these bacteria develop resistance towards AMP. Besides AMP, the results revealed higher resistant among coliforms towards CIP, CX, and NA. In this study, altogether 11 MDR isolates were detected. Among them, 7 were from raw samples and rest were from pasteurized samples. 9 MDR were detected in the study of Rai *et al.* (2017).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Materials

The materials and equipment required for this study are listed in Appendix I.

3.2 Methods

3.2.1 Study site/ Sampling site

This study was conducted in Kathmandu district. Sampling and analysis of the milk samples were performed during January 2021 to May 2022. It is densely populated district of Nepal with 19,88,606 people in 2022.

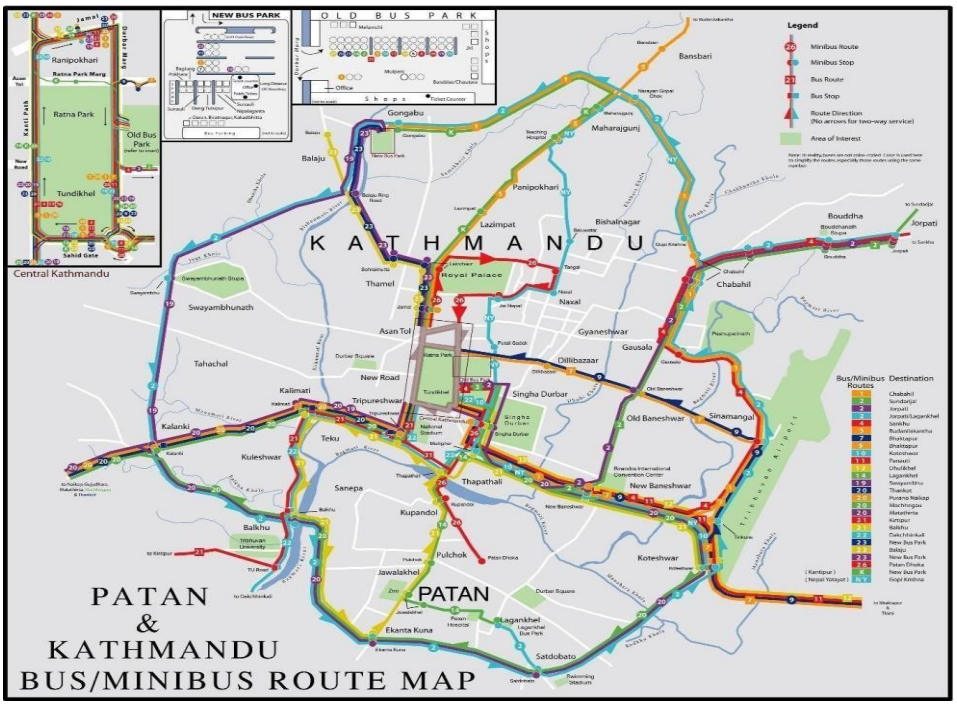


Figure 2 Map of study area

Pasteurized milk was collected from local vendors of Kathmandu while raw milk was collected from Budhanilkantha area according to the availability of commercial farm and small stock holders.

3.2.2 Study design and data collection

Observational and cross-sectional study were applied. Primarily, data was collected by the experimental observations in the laboratory by taking different samples and secondarily, the data was contradicted with the published documents.

3.2.3 Sample collection and transportation

Altogether 30 milk samples (15 raw and 15 pasteurized) were taken for this study. Raw samples were collected from different farm of Budhanilkantha in sterilized screw capped tubes taken in icebox. Samples were processed promptly within 2 hours. Pasteurized samples of different brands were collected from different local vendors and taken to the laboratory. It was labelled well and details (such as solid not fat, fat content, etc.,) of samples were noted.

3.2.4 Serial dilution

After collection of pasteurized samples, cover was cleaned with 99.9% alcohol and were serially diluted up to 10^{-6}.

In contrary, raw samples were collected in sterile screw capped tube which was taken in the icebox and then it was brought to room temperature in laboratories. The samples were serially diluted up to
10^{-8}.

3.2.5 Adulteration tests and MBRT

10 mL of the samples were collected for MBRT and quality of the samples were detected quantitatively. 5 mL and 3 mL of the samples were collected for soda and starch adulteration tests respectively (Appendix III).

3.2.6 Enumeration of bacteria

After the sample was prepared, 1mL of each dilution of samples were transferred to the sterile petri-plate and Violet Red Bile Agar (VRBA) (at around 45℃) was poured into the respective petri- plates for the enumeration of coliforms. Double layering of the media was done in case of VRBA to maintain facultatively anaerobic condition.

Then, the plates were incubated at 37℃ for 24 hours for total coliform count and at 44℃ for faecal coliform count (Cheesebrough, 2006). Enumeration was done and result was interpreted accordingly based on DFTQC and BIS guidelines.

3.2.7 Isolation and identification

Isolated colonies from VRBA were transferred to nutrient broth and incubated for 4 hours and each sample were taken for gram staining and based on gram reaction the samples were transferred to biochemical test set up and also sub-cultured on nutrient agar and MacConkey agar and both of them were incubated at 37℃ for 24 hours. After 24 hours the results were noted and from nutrient agar plate biochemical tests such as IMViC, O/F, TSI, SIM, catalase and oxidase were performed. The results were interpreted.

3.2.8 Antimicrobial Susceptibility Pattern of the identified isolates.

In vitro antimicrobial susceptibility pattern of the identified isolates towards different antibiotics was performed by modified Kirby Bauer disc diffusion method on to Muller Hinton Agar (MHA) and zone size was interpreted by using CLSI guideline (2014). For this, 12 different antibiotic discs of known contents were used namely, Ciprofloxacin (5mcg), Ceftriaxone (30mcg), Ampicillin (10mcg), Cefoxitin (30mcg), Nalidixic Acid (30mcg), Nitrofurantoin (300mcg), Tetracycline (30mcg), Piperacillin/ Tazobactam (100mcg), Co- Trimoxazole (25mcg), Levofloxacin (5mcg), Chloramphenicol (30mcg) and Amikacin (30mcg).

3.2.9 Quality control

ATCC culture of *E. coli* 25922, *Klebsiella* spp. 700603 and *S. aureus* 25923 were used for AST and its zone of diameter was compared with the standard given by CLSI. Instruments were optimized. Sterile conditions were maintained throughout the processing of samples to avoid cross contamination.

3.2.10 Data analysis

Collected data were entered in MS-excel. Descriptive analysis was done using bar graph, pie-chart and tabular forms. Statistical analysis was done using SPSS (Version-20) software. Mean and Standard deviation were calculated from the bacterial count. Comparison of mean was done using 95% of level of significance.

CHAPTER 4

4. RESULTS AND DISCUSSION

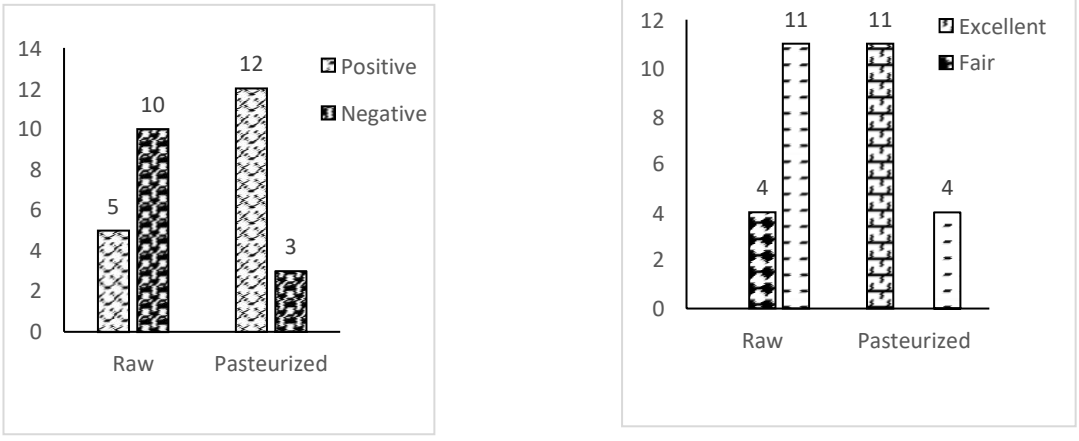
4.1 Results

In this study, altogether 30 samples (pasteurized milk and raw milk) were taken for determining the microbial load and quality of the milk consumed.

4.1.1 Adulteration tests and MBRT of milk samples

Out of 30 samples for soda adulteration test 17(56.67%) samples were positive for soda. The sample-wise result of soda adulteration test is shown in figure 3(a). Starch adulteration was not observed in any of the samples. While for Methylene blue reduction test (MBRT) for 15 raw milk samples, 11samples (73.33%) were found to be "good" quality while 4 samples (26.67%) were of "fair" quality based on the interpretation criteria of DFTQC (Appendix IV).

While for pasteurized milk samples, 11 samples (73.33%) were of "excellent" quality while 4 samples (26.67%) were of "good quality based on the interpretation criteria for MBRT, DFTQC standard. The sample-wise result of MBRT is shown in figure 3(b).



(a) (b)

Figure 3 Sample wise result of (a) Soda adulteration test (b) MBRT

4.1.2 Microbial quality of Raw milk and Pasteurized milk

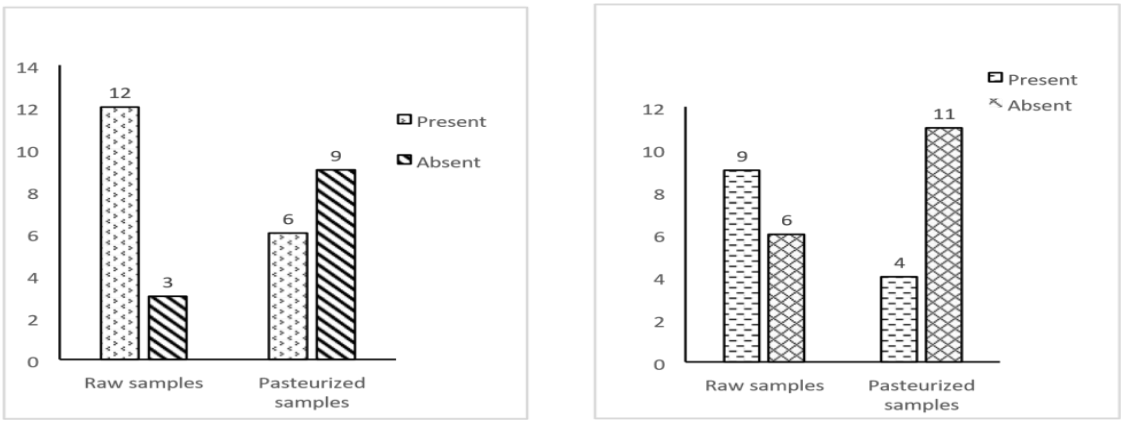
4.1.2.1 Total Coliform Count (TCC)

The TCC of all the samples were ranged from 0.0037×10^5 CFU/mL to 0.56×10^5CFU/mL and the mean of 0.1558×10^5 CFU/mL. In raw samples, the TCC ranged from 0.0037×10^5 CFU/mL to 0.5625×10^5 CFU/mL and the mean of 0.179×10^5 CFU/mL. In pasteurized samples, the TCC ranged from 0.0086×10^5 CFU/mL to 0.245×10^5 CFU/mL and the mean of 0.09005×10^5 CFU/mL. Sample-wise distribution of total coliforms is shown in figure 4(a).

4.1.2.2 Faecal Coliform Count (FCC)

Faecal coliform counts of the samples were ranged from 0.0036×10^5 CFU/mL to 0.19×10^5 CFU/mL and the mean of 0.075×10^5 CFU/mL.

In raw samples, FCC ranged from 0.0036×10^5 CFU/mL to 0.19×10^5 CFU/mL and the mean of 0.0975×10^5 CFU/mL whereas in pasteurized samples, FCC ranged from 0.0066×10^5 CFU/mL to 0.087×10^5 CFU/mL and the mean was 0.048×10^5 CFU/mL. Sample-wise distribution of total coliforms is shown in figure 4(b).



(a) (b)

Figure 2 Distribution of the coliforms in the raw and pasteurized samples showing (a) TCC and (b) FCC.

4.1.3 Isolation and identification of milk isolates

Altogether 31 isolates were identified in this study. Among them 21 (67.74%) were from raw samples and rest (32.26%) were from pasteurized samples. In raw samples, 12 (57.14%) isolates belonged to TCC and 9 (42.86%) were found to be FCC. On the other hand, 6 (60%) isolates were belonged to TCC and 4 (40%) were FCC, in pasteurized sample. Among the total isolates, 17 (54.84%) isolates were identified as *Klebsiella* spp., 13 (41.94%) were *Escherichia coli* and 1 (3.22%) was *Citrobacter* spp. Different isolates and their distribution in both the samples are given in figure 5.

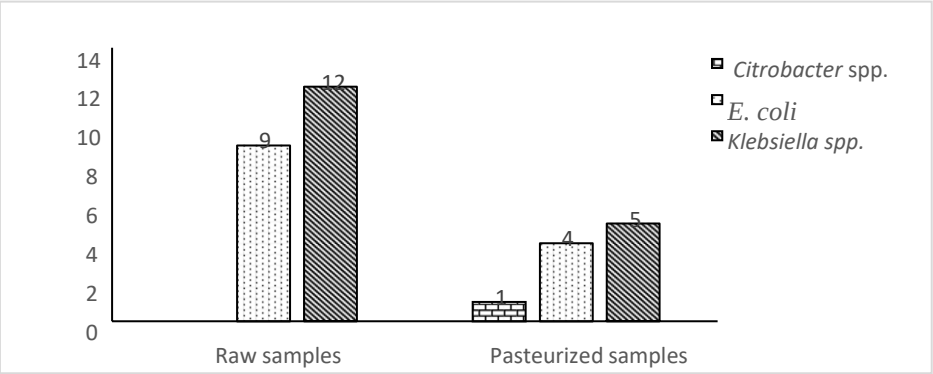


Figure 5 Distribution of identified isolates in the samples.

4.1.4 Antibiotics Susceptibility Pattern of Identified Isolates

In Antibiotic susceptibility pattern of identified isolates, 12 different antibiotic discs of known contents were used namely, Ciprofloxacin (CIP), Ceftriaxone (CTR), Ampicillin (AMP), Cefoxitin (CX), Nalidixic Acid (NA), Nitrofurantoin (NIT), Tetracycline (TE), Piperacillin/ Tazobactam (PIT), Co- Trimoxazole (COT), Levofloxacin (LE), Chloramphenicol (C) and Amikacin (AK). Altogether 31 isolates were processed for the determining Antibiotic Susceptibility Pattern. Here, intermediate isolates were included in resistant. Mean value of each antibiotic according to sample is shown in Table No. 2.

Table No. 2 Mean value of zone of inhibition each antibiotic according to sample.

Antibiotics	Samples	N	Mean ± S.D
CIP	Raw	21	27.05 ± 2.958
	Pasteurized	10	27.70 ± 2.541
CTR	Raw	21	28.76 ± 2.047
	Pasteurized	10	26.50 ± 6.005
AMP	Raw	21	7.67 ± 3.864
	Pasteurized	10	7.70 ± 3.653
CX	Raw	21	12.86 ± 6.923
	Pasteurized	10	16.20 ± 5.770
NA	Raw	21	18.90 ± 8.467
	Pasteurized	10	22.20 ± 6.512
NIT	Raw	21	20.33 ± 2.763
	Pasteurized	10	21.60 ± 2.675
TE	Raw	21	22.48 ± 2.786
	Pasteurized	10	22.90 ± 2.685
PIT	Raw	21	25.62 ± 2.133
	Pasteurized	10	25.70 ± 1.160
COT	Raw	21	26.57 ± 2.249
	Pasteurized	10	25.20 ± 6.989
LE	Raw	21	24.95 ± 2.439
	Pasteurized	10	24.90 ± 2.807
C	Raw	21	24.81 ± 2.421
	Pasteurized	10	23.80 ± 4.131
AK	Raw	21	19.38 ± 1.658
	Pasteurized	10	19.10 ± 1.524

Out of total isolates from both the samples, 31(100%) were sensitive against TE, 30 (96.77%) against NIT, PIT, COT, C and AK, 24 (77.41%) were sensitive against CIP and NA. 29 (93.55%), 28(90.32%), 5 (16.13%) and 2 (6.45%) were sensitive against CTR, LE, CX and AMP respectively.

Sample-wise antibiotic susceptibility patterns of milk isolates are given in Figure 5 and figure 6.

Among the identified isolates, 11 (35.5%) of them showed resistant against three or more class of antibiotics, which is term as MDR (Multi- Drug Resistant). Among these isolates, 7 of them were from raw samples and rest from pasteurized samples.

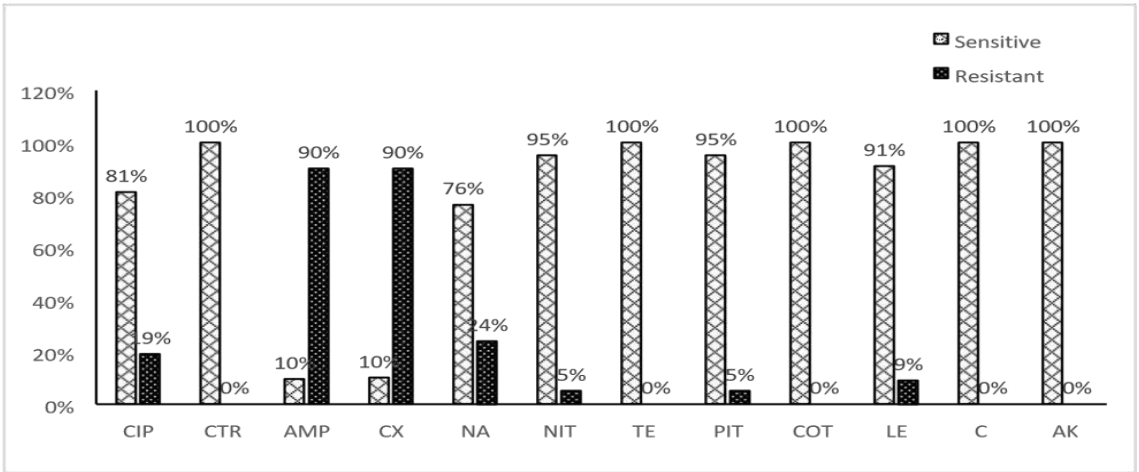


Figure 5 Antibiotic susceptibility pattern of isolates from raw samples.

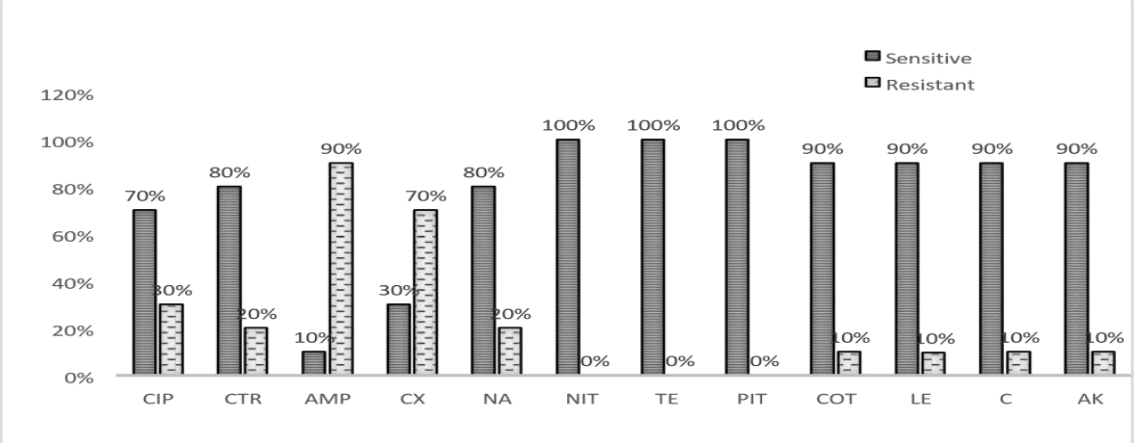
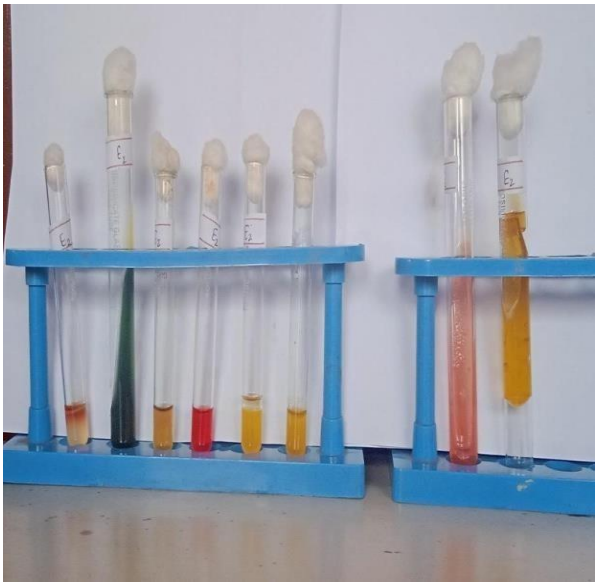


Figure 6 Antibiotic susceptibility pattern of isolates from pasteurized samples.

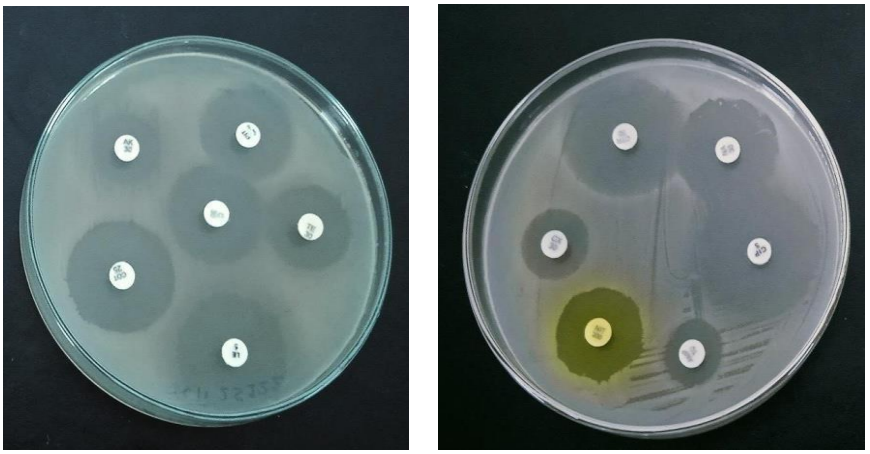
PHOTOGRAPHS

Photograph 1 Milk sample showing growth of *Klebsiella* spp. colonies on MacConkey's Agar.

Photograph 2 Milk sample showing growth of *E. coli* colonies on MacConkey Agar.



Photograph 3 Biochemical test results of *E. coli* (Right to left: TSI- acid/acid, H_2S^-, gas^+, Urease-negative, O/F- Fermentative, MR- positive, VP- negative, Citrate utilization- negative, SIM- Indole and motility positive, H_2S- negative).



Photograph 4 Antibiotic susceptibility testing (Right figure, LE, AK, TE, PIT, C and COT and in the left, CIP, CTR, NIT, CX, AMP, NA).

(a) (b)

Photograph 5 Results showing (a) MBRT and (b) Soda adulteration tests

Photograph 6 Sample processing in the Microbiology laboratory of Amrit Campus.

4.2 DISCUSSION

Thirty milk samples (15 raw and 15 pasteurized) were taken for this study. Samples were prepared for the test such as adulteration test (Starch and Soda), MBRT, TBC, and TCC. Samples were processed following standard protocol (Appendix III).

In general, milk is adulterated with soda, starch, table sugar and water. But in this study, adulteration with starch was not observed in any of the sample. However, soda adulteration was found in most of the samples (56.67%) in this study. Contrasting result was reported by Barham, G.S. *et al*. (2015) showing lower Soda adulteration and higher starch adulteration. Soda is generally used as a neutralizer. Therefore, farmer's may have added soda to avoid milk spoilage during transportation. During Methylene Blue Reduction Test (MBRT) of this study, 11 (36.67%) of the total samples were found to be of "Excellent" quality, 15 (50%) were "good" quality and 4 (13.33%) were of "Fair" quality according to DFTQC and BIS guideline. Unlike this study, Pawar, J. and Kalpita, M. (2021) reported that the higher extents of the samples were of "fair" quality and few samples were of "good" quality according to BIS (Bureau of Indian Standard) 1992. Along with some bacterial loads, samples also contained coliforms which are also known as indicator organisms. According to BIS guidelines and DFTQC guidelines, coliform count should be absent in 1:10 dilution but in this study, coliforms were observed in most (60%) of the samples. In this study, TCC were higher in raw samples and lower in pasteurized samples. Similar result was reported in the study of Uddin *et al.* 2014.

In the raw samples, TCC of most of the samples (80%) were found to be above the range given by BIS guidelines in this study. Similar study was reported by Sarita *et al.* (2020). This may be due to the entrance of the coliforms via water. Coliforms are found in the soil, in water, and in human or animal waste. There is strong evidence that sewage has a role in coliform distribution (Uddin *et al.* (2014)).

In the pasteurized samples, TCC of 40% were found to be above range given by BIS guidelines, in this study. Unlike this study, Shrestha, P (2012), Shrestha, S (2017), Sarita *et al.* (2020), and Rai S *et al.* (2020) reported higher extents of their samples were above the range based on the same guidelines. This may be due to the defect in pasteurization process or flaws in post pasteurization such as defects in pipe lines, packaging material, coliforms may be detected in the pasteurized samples. Efficiency of pasteurization should be analyzed in order to prevent the entry of bacteria in the sample.

In this study, faecal coliform count was done and found to be higher for the raw samples (60%) and lower in the pasteurized samples (26.67%). The faecal coliforms were present in approximately half of the samples (43.33%) in both the samples. Nowadays, milk and milk products are also containing faecal coliforms. Unlike this study, Phuyal, S (2019) reported higher (54.2%) prevalence of thermotolerant coliforms from paneer samples than this study. This may be due to the entry of coliform via water, dung or soil.

In the raw samples, 60% of the samples were found to surpassed the guidelines given by BIS (1992) for coliforms. Similar to this study, Ahmed M Hammad *et al* (2022) reported 65% of their samples were unsuitable for consumption as they exceeded the standards for coliforms. Among 31 isolates of this study, *Klebsiella* spp. (54.84%) was predominant followed by *E. coli* (41.94%) and *Citrobacter* species (3.22%). This prevalence is lower when compared to the findings of Shunda, D *et al.* (2013), Chye F. Y *et al* (2004) and Ligathurai, S (2013) *et al.* who reported prevalence of *E. coli* from milk samples of 63%, 70% and 65% respectively. Unlike this study, Adil M. A. Salman and Iman M. Hamad (2011), Sarita *et al.* (2020) and Nigatu Disassa *et al.* (2017) reported lower prevalence of *Escherichia coli* in their study where they stated prevalence of *E. coli* as 32%, 40.6% and 33.9% respectively. The variation was seen in prevalence in different studies may be due to difference in sample size, farming system, farm size, milking equipment, milking technique, geography, ecology, duration of milk transportation, and hygienic conditions (Nigatu Disassa *et al.* (2017).

In this study, total coliform present was higher in the raw samples than the pasteurized samples. Unlike this study, Acharya *et al*. (2017) reported the higher coliform count of both the samples than this study. The prevalence of frequent *E. coli* in their study may be due to the entrance of the bacteria via water, soil, dung, and environment. The presence of higher extents of *Klebsiella* spp. in this study may be due to the distribution of these species in environments.

Altogether 31 isolates were processed for the determining Antibiotic Susceptibility Pattern of identified isolates. Regarding the antibiotic susceptibility test of the identified isolates, the result showed an emerging antibiotic resistant among the isolates. Most of the isolates were resistant to AMP which was in compliance with the findings of Rai *et al*. (2020) and Badri *et al*. (2017). Beside AMP antibiotic, the raw samples showed higher sensitivity towards CTR, TE, C, AK, NIT, PT, LE, CX, CIP, NA respectively in descending order. However, pasteurized samples showed higher sensitivity towards NIT, TE, PIT, COT, LE, C, AK, NA, CTR, CIP respectively in the descending order.

On the other hand, isolates from raw samples showed the 100% sensitivity towards CTR, TE, COT, C, and AK. Similar result was stated by Acharya *et al*. (2017) in their study where they reported 100% sensitivity of isolates towards COT and C. 95% were sensitive towards NIT and PIT, 9.5% towards CX and AMP, 90.5%, 81% and 76.2% towards LE, CIP and NA respectively in this study.

Unlike this study, Uddin *et al.* (2011) reported in his study that all the *E. coli* isolated from raw sample exhibited 100% resistant against Nalidixic Acid (NA).

In this study, isolates from pasteurized samples showed the 100% sensitivity towards NIT, TE, and PIT. Similar result was stated by Acharya et al. (2017) who reported all the *E. coli* isolates were sensitive towards PIT. 90% were sensitive towards COT, LE, C and AK, 80% towards CTR and NA and 70%, 30% and 10% towards CIP, CX and AMP respectively in this study. Unlike this study, Nigatu Disassa *et al.* (2017) in their study reported that most of the *E. coli* isolates were resistant to tetracycline (81.8%) and cefoxitin (54.5%) which was unlike this study.

This emerging antibiotic resistance among the isolates was observed higher in raw milk sample compared to pasteurized milk sample. Since the exposure to the environment is more in raw milk than pasteurized milk, the chances of the resistant isolates finding its way to milk is more likely. Further the extensive misuse of antibiotics for the treatment of farm animals may have created selective pressure and resultant isolates. This emerging resistance may lead to treatment failure of the last resort drug. Thus, routine monitoring of resistant profile of milk pathogens should be implemented in order to properly diagnose and treat milk-borne infections effectively, along with the assessment of microbial quality of milk with the purpose of safeguarding the consumers.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

TCC and FCC confirmed the presence of coliform in both the samples. Antibiotic susceptibility pattern showed higher resistant towards some antibiotics. Therefore, milk sold in the Kathmandu valley cannot be considered as good quality based on the results that they are adulterated and antibiotic resistant coliforms are detected in those samples. So, regular monitoring of milk quality and milk isolates should be done by concerning authorities (HACCP or ISO 22000) to avoid developments of significant resistant to the available drugs.

5.2 Novelty and National Prosperity aspects of Project work

Microbial quality of the food samples has been assessed from long time but due to various factors such as careless behaviour of the employee, less capable people in the related field and so on the consumers are still not getting the quality product for the consumption. The production sites are not following the guidelines and keep selling their products in expense of public health due to which the regularly consumed food products such as milk, and the rest are released with the hazardous microbial community. This study assessed the microbial quality of milk and suggest people for the proper selection of food products. The sample of this study contained total coliform and faecal coliform which is not assessed in routine analysis by the production site and their presence is threat in the aspects of public health. This will help in improving the public health aspect at the national level. Besides this, the heat resistant faecal coliform present nowadays in pasteurized and raw milk is the greater issue at the public level and deteriorating the consumer's health drastically.

5.3 Limitations of the work

i. The sample size was taken small due to the lack of sufficient time.

ii. This project was limited by the financial burden.

iii. Extended analysis (like molecular analysis) could not be done due to limited resources and

time.

5.4 Recommendation for further work

Based on the findings of the present study, the following recommendations are made:

i. A high standard of cleanliness should be maintained at all times in the farm area to reduce microbial contamination in collected milk. Milking equipment and transportation container should be cleaned thoroughly in order to avoid cross-contamination.

ii. Sick animals or animals under treatment should be kept in isolation in the farm to minimize the spread of contagious disease. Also, milk from such animals should not be sold or consumed by anyone.

iii. More research work has to be conducted in different areas of Kathmandu with the aim of quantifying the magnitude of milk-borne pathogens as it may be present in small-scale livestock keepers' communities and developing resistance to antimicrobial agents.

iv. Concerned authorities should conduct frequent inspection of the marketed milk to check whether they meet the minimum legal standards and should monitor the overall hygienic condition surrounding the production and handling of milk.

v. Good manufacturing practice, good hygiene practice and hazard analysis and critical control

point should be implemented in dairy industry to prevent the contamination of dairy products.

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APPENDIX I

LIST OF MATERIALS

Equipment	Company
Conical flasks	BOROSIL, India
Refrigerator	Express Cool, LG
Pipette	BOROSIL, India
Glass tubes and pipettes	BOROSIL, India
Petri plate	BOROSIL, India
Hot Air Oven	Ambassador
Autoclave	Life
Microscope	COSLAB
Incubator	Memmert
Ice box	Marina, 2S
Electric balance	PHOENIX

1. Microbiological Media (Hi Media) Basal Media

Nutrient Agar Nutrient Broth Plate Count Agar

2. Selective and Differential

Violet Red Bile Agar (VRBA)

3. Other Media

Muller Hinton Agar (MHA)

Biochemical Media

1. SIM Media
2. TSI Media
3. MR-VP Media
4. Simmon Citrate Agar Media
5. Urease Agar Media
6. Hugh and Leifson's Media

Reagent and Chemical Used for Identification of Organisms

7. Crystal Violet solution
8. Gram's Iodine
9. Acetone Alcohol
10. Safranin
11. 3% Hydrogen peroxide solution
12. 1% Tetramethyl -p-phenylenediamine dihydrochloride
13. Kovac's Reagent
14. Normal Saline

APPENDIX II

COMPOSITION AND PREPARATION

1. Diluent and Culture Media

i. Normal Saline, 8.5 g/l

Composition	Grams/L
Sodium Chloride	8.5
Distilled water	1 litre

Preparation: 8.5g of sodium chloride was weighed and transferred to a leak-proof bottle pre-marked to hold 1 litre. Then, distilled water was added up to 1 litre mark, and mixed until the salt was fully dissolved. The bottle was labelled and stored at room temperature.

ii. Violet Red Bile Agar (VRBA)

Composition	Grams/L
Peptone	7.00
Yeast extract	3.00
Sodium Chloride	5.00
Bile salts mixture	1.500
Lactose	10.00
Neutral red	0.030
Crystal violet	0.002
Agar	15.00

Final pH (at 25℃): 7.4 ± 0.2

Preparation: 41.53g of agar was weighed and mixed in 1000mL of distilled water in the conical flasks. It was boiled and the medium was dissolved. Then, it was sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes.

iii. Nutrient Agar

Composition	Grams/L
Peptic Digest of animal tissue	5.00
Sodium Chloride	5.00
HM peptone B#	1.50

Yeast Extract	1.50
Agar	15.00

Final pH (at 25℃): 7.4 ± 0.2

Preparation: 28g of the medium was suspended in 1000mL distilled water and boiled completely. It was sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes.

i. Nutrient Broth

Composition	Grams/L
Peptic Digest of animal tissues	5.00
Yeast Extract	1.50
Beef Extract	1.50
NaCl	5.00

Final pH (at 25℃): 7.4 ± 0.2

Preparation: 25 grams of media was suspended in 1000mL purified/distilled water and heated if necessary, to dissolved the medium completely. Then, the medium was sterilized by autoclaving at 10lbs pressure (115℃) for 30 minutes or alternatively at 15lbs pressure (121℃) for 15 minutes or as per validated cycle.

v. Muller Hinton Agar (MHA)

Composition	Grams/L
Beef infusion	300.00
Acid of casein hydrolysate	17.50
Starch	1.50
Agar	17.00

Final pH (at 25℃): 7.3 ± 0.1

Preparation: 38g of the medium was suspended in 1000mL distilled water and boiled to dissolved completely. The medium was sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes. After that it was cooled down to 45-50℃ and mixed well. Then, it was poured into sterile petri-plates.

Biochemical media

I) MR-VP Broth

Composition	Grams/L
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Agar	2.0

Final pH (at 25℃) 6.9± 0.2

Preparation: 17 grams of MR-VP medium was suspended in 1000mL distilled water and boiled to dissolved completely. Then it was dispensed in tubes and sterilized by autoclaving at 15lbs pressure (121˚C) for 15 minutes.

II) OF Basal Medium

Composition	Grams/L
Casein enzyme hydrolysate	2.00
Sodium chloride	5.00
Dipotassium Phosphate	0.30
Bromothymol Blue	0.08
Agar	2.00

Final pH (at 25℃) 6.8± 0.2

Preparation: 9.38 grams of O/F Basal Medium was suspended in 1000mL distilled water and boiled to dissolved completely. The solution was then dispensed in 100mL amounts and sterilized by autoclaving at 15lbs pressure (121℃) for 15 minutes. To first 100 mL of sterilized basal medium., 10mL of sterile 10% dextrose solution was added aseptically. To second 100mL, 10 mL sterile 10% lactose solution was added. To third 100mL, 10 mL sterile 10% saccharose solution was added. The solution was mixed and dispensed aseptically in 5mL amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation.

III) Sulphide Indole Motility (SIM) agar

Composition	Grams/L
Beef Extract	3.00
Peptic Digest of animal tissue	30.00
Peptonized ion	0.20
Sodium Thiosulfate	0.025
Agar	3.00
Distilled water	1000

Final pH (at 25℃) 7 .3± 0.2

Preparation: 36.23g of the medium was suspended in 1000mL distilled water and heated to boil. The medium was dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121˚C) for 15 minutes.

IV) Simmon's Citrate Agar

Composition	Grams/L
Magnesium sulphate	0.20
Ammonium Dihydrogen Phosphate	1.00
Dipotassium Phosphate	1.00
Sodium Chloride	5.00
Sodium Citrate	2.00
Bromothymol blue	0.08
Agar	15.00

Final pH (at 25℃) 6.8± 0.2

Preparation: 24.28g of the medium was suspended in 1000mL of the distilled water and heated to boiling to dissolve the medium completely. The medium was then dispensed in tubes. And was sterilized by autoclaving at 15lbs pressure (121˚C) for 15 minutes. The medium was allowed to set in slope for the development of the butt and slant.

V) Urea Agar Base

Composition	Grams/L
Peptic Digest of animal tissue	1.00
Dextrose	1.00
Monopotassium Phosphate	0.80
Dipotassium Phosphate	1.20
Sodium Chloride	5.00
Phenol Red	0.012
Agar	15.00

Final pH (at 25℃) 6.8± 0.2

Preparation: 24g of the media was suspended in 950 mL of distilled water and dissolved completely by boiling. Then it was sterilized by autoclaving at 10lbs pressure (115℃) for 20 minutes. It was then cooled to 45-55℃ and it was poured on to petri-plate.

VI) Triple Sugar Iron (TSI Agar)

Composition	Grams/L
Peptic Digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast Extract	3.00
Beef Extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Ferrous Sulphate	0.20
Sodium Chloride	5.00
Sodium Thiosulphate	0.30
Phenol Red	0.024
Agar	12.00

Final pH (at 25℃) 7.4 ±0.2

Preparation: 64.52g of the medium was suspended in 1000mL distilled water and heated to boiling point to dissolve the medium completely. The medium was dispensed into test tubes. It was then sterilized by autoclaving at 15lbs pressure (121˚C) for 15 minutes. The medium was allowed to set in slope for the development of the butt and slant.

Staining reagents

1. Crystal violet (CV)

Composition	Contents
Crystal violet	20.00g
Ammonium Oxalate	9.00g
Ethanol	95.00mL
Distilled Water	1000mL

Preparation: In a piece of clean paper, 20g of crystal violet weighed and transferred to a clean brown bottle. Then 95mL of ethanol was added and mixed until the dye completely dissolved. Then 9g of ammonium oxalate was weighed and dissolved in about 200mL distilled water.

Then it was added to stain. Finally, the volume was made 1 litre by adding distilled water.

2. Iodine solution

Composition	Contents
Potassium Iodide	2g
Iodide	1g
Distilled water	300mL

Preparation: To 300mL of distilled water, 2 g of potassium iodide and 1 g of iodine was mixed until it is dissolved completely. Then, it was transferred in a brown bottle and stored in the dark at room temperature.

3. Acetone alcohol (decolourizer)

Composition	Contents
Acetone	500mL
Ethanol	475mL
Distilled water	25mL

Preparation: To 25 mL of distilled water, 475g of absolute alcohol was added mixed and transferred in a clean bottle. Then immediately, 500 mL of acetone was added to the bottle and mixed well.

4. Safranin

Composition	Contents
Safranin	10.0 mL
Distilled water	100 mL

Preparation: To 10 mL of safranin solution, 100 mL of distilled water was added and mixed well until safranin dissolved completely.

Biochemical reagents

1. Catalase Reagent (3% H_2O_2)

Composition	Amount
Hydrogen peroxide	3 mL
Distilled water	97 mL

Preparation: To 97 mL of distilled water, 3 mL of hydrogen peroxide was added and mixed well.

2. Oxidase Reagent

Composition	Contents
Tetramethyl-p- phenyl diamine dihydrochloride	1 g Distilled
water	100 g

Preparation: This reagent solution was made by dissolving 1 g TPD in 100 mL distilled water.

The strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

3. Kovac's Reagent

Composition	Contents
Amyl alcohol	75 mL
P-Dimethyl amino benzaldehyde	5.0 g
Concentrated Hydrochloric Acid	25 mL

Preparation: 5 g P-Dimethyl Amino benzaldehyde was dissolved in 75 mL amyl alcohol. Then 25 mL of hydrochloric acid was added slowly to the mixture and mixed well. The reagent was then stored in a glass-stoppered bottle in refrigerator.

4. Methyl Red solution

Composition	Contents
Methyl Red	0.04 g
Ethyl alcohol	40.0 mL
Distilled water	60 mL

Preparation: 0.04 g of methyl red was dissolved in 40 mL of ethyl alcohol and transferred to a clean brown bottle. To this 60 mL of distilled water was added and mixed well.

5. Barrit's Reagent

Solution A

Composition	Contents
Alpha-naphthol	5.00 g
Absolute alcohol	100 mL

Preparation: 5 g of alpha-naphthol was dissolved in 100 mL absolute alcohol and transferred to a clean bottle and final volume was made to 100 mL by adding distilled water.

Solution B

Composition	Contents
Potassium hydroxide	40 g
Distilled water	100 mL

Preparation: 40 g of potassium hydroxide was dissolved in 25 mL of distilled water and transferred to a clean bottle and final volume was made to 100 mL by adding distilled water.

1. Preparation of Turbidity Standard equivalent to McFarland 0.5

To standardize the inoculum density for a susceptibility test, a BaCl turbidity standard, equivalent to 0.5 McFarland standard was used.

1. 1% v/v solution of Sulfuric acid was prepared by adding 1 mL of concentrated sulfuric acid to 99 mL water.
2. 1% w/v solution of barium chloride was prepared by dissolving 0.5 g of dehydrate barium chloride ($BaCl_2.2H_2O$) in 50 mL of distilled water.

3. 0.6 mL of the barium chloride was added to 99.4 mL of the sulfuric acid solution and mixed.
4. A small volume of the turbid solution was then transferred to a screw capped bottle.

2. Preparation of the Methylene Blue Dye (1:30000)

For the preparation of Methylene blue reagent, 0.1 gram of methylene blue dye was completely dissolved in 9mL of sterile distilled water after which volume of the solution was 10-fold serially diluted, twice, and 3- fold, once, using sterile distilled water to obtain 1:30000 concentration of the dye. The dye was then stored in sterile amber coloured bottle in dark to prevent photooxidation.

APPENDIX III

PROCEDURE

I. Enumeration of bacteria from milk

1. 1mL milk sample was at first pipetted out by using sterile pipette. Then, it was poured into sterile diluent in a test tube.
2. Then, 1 mL was pipetted from 10^{-1} to 10^{-2} diluent.
3. Similarly, from 10^{-2}, 1 mL was pipette to 10^{-3} diluent. Same process was repeated up to 10^{-6} (for pasteurized milk) and up to 10^{-8} (for raw milk).

i. Procedure for Pour Plate Technique:

1. Milk was serially diluted up to (10^{-6} / 10^{-8}) dilution.
2. 1mL from 10^{-1} to (10^{-6} / 10^{-8}) dilutions were pipetted out aseptically with the help of sterile pipette into sterile petri-dishes.
3. Then, the molten VRBA was cooled down to 45℃ and approximately 15 mL of VRBA was poured into each petri-plate containing the diluted sample.
4. Then, the sample and agar medium were mixed by rotating gently to ensure uniform distribution.
5. The plates were then allowed to solidify.
6. The plates were then incubated for 24-48 hours at 37℃ and 44℃ in the inverted position (lid on bottom).
7. After incubation, all the plates were observed for the appearance of bacterial colonies.
8. The number of colonies were counted in the plates.
9. Then, the CFU/mL of bacteria was calculated.

ii. Procedure for Streak Plate Technique

1. At first, inoculating loop was sterilized. Then, the loop was introduced into the broth and one loopful of culture was withdrawn from test tubes.
2. Then, the mouth of the test tube was flamed and cotton wool was then replaced.
3. The petri-plate was then lifted with the left hand and held at an angle of 60°C.
4. The inoculum was placed on the agar surface and streaked from side to side in parallel lines across the surface of area.

5. Loop was then re-flamed and cooled. Petri-plate was turned to 90° C. Loop was touched to a corner of the culture media in area 1 and the inoculum was streaked across the agar in area 2.
6. The rest of the agar surface was then used to complete the quadrant streaking.
7. Lid of the petri-plate was replaced after streaking was completed and the loop was sterilized by flaming.
8. Then the plates were incubated for 24-48 hours at 37℃ in an inverted position (lid on bottom).

II. Gram Staining and Biochemical Tests Procedure for Gram staining:

1. A clean grease free slide was taken and a thin smear of the colony of organism was made on the slide. The slide was air dried and then heat fixed.
2. The smear was covered with crystal violet for 1 minute and then washed with water.
3. Then the slide was covered with Gram's iodine for 1 minute and washed.
4. Then the acid alcohol was added to the slide for 15 seconds and then it was rinsed with water gently.
5. Finally, the slide was covered with safranin for 1 minute and washes.
6. The slide was blot dry or air dried and observed under microscope under oil immersion.

Procedure for Catalase test:

1. Using sterile applicator stick, a portion of colony was transferred to the surface of clean, dry glass slide.
2. A drop of 3% H_2O_2 was placed on the glass slide and mixed.
3. Then, effervescence of the gas was marked by bubbles in case of positive result.

Procedure for Oxidase test:

1. A piece of filter paper was soaked with the oxidase reagent 1% tetramethyl-p phenylenediamine dihydrochloride.
2. A small portion of bacterial colony was taken with the help of sterile wooden stick and rubbed on the paper.
3. The paper was observed for the development of purple colour.

Procedure for Oxidative/Fermentative Test:

1. Two tubes containing O/F medium were taken and the organism was stabbed into both media using sterile inoculating wire.
2. One of the tubes was sealed with paraffin oil to create anaerobic condition.
3. Both the tubes were incubated at 37℃ and observed for colour change in both the tubes.

Procedure for Sulphide Indole Motility (SIM) test:

1. The organism was stabbed into the SIM medium with the help of sterile inoculating wire and incubated at 37℃.
2. After proper incubation, 4-8 drops of Kovac's reagent were added to the tube and mixed, and let it stand for a while.
3. The tube was observed for the development of cherry red colour on the surface, as well as blackening of the medium and spreading of the bacterial growth.

Procedure for Methyl Red (MR) test:

1. MR-VP broth was aseptically inoculated with the organism with the help of sterile inoculating loop and incubated at 37℃.
2. Then, 5-6 drops of Methyl red reagent were added to the tube.
3. The positive test is indicated by the development of red colour of the indicator.

Procedure for Voges Proskauer (VP) test:

1. The bacterial suspension was inoculated aseptically into the MR-VP broth tube and incubated at 37℃ for 24 hours.
2. Then the Barrit's reagent I and II were added in the ratio of 3:1 and the tubes were shaken.
3. The tube was observed for the development of red colour after incubation for 2030 minutes aerobically.

Procedure for Citrate utilization test:

1. The organism was streaked aseptically on the Simmons citrate agar and incubated at 37℃ for 24-48 hours.
2. Change in the colour of the medium was observed.

Procedure for Triple sugar iron agar test (TSIA):

1. Test organism was inoculated in the TSIA slant by stabbing butt first and streaking on the surface of the slant using sterile inoculating wire.
2. The TSIA slant was then incubated at 37℃ for 18 hours.
3. Then the colour change in the butt and slant was observed along with gas production and H_2S production.

Urea Hydrolysis Test:

1. The test organism was streaked on the surface of the urease agar slant and incubated at 37℃ for 24-48 hours.
2. The change in colour of the medium was observed.

III. Antibiotic Susceptibility Testing:

1. Using a sterile inoculating loop, single isolated colony of the test organism was inoculated and emulsified into 3-4 mL of nutrient broth and was incubated at 37℃ for 4 hours.
2. Then, in a good light, the turbidity in a prepared inoculum was compared with 0.5 McFarland standard.
3. Then sterile cotton swab was dipped into the broth containing culture. Excess was removed by pressing and rotating the swab against the side of the tube above the level of suspension.
4. Then, it was streaked evenly over the surface of the MHA medium in three directions, rotating the plate approximately 60° to ensure even distribution.
5. With the petri-dish lid in place, the surface of the agar was allowed to dry for 3-5 minutes.
6. The antibiotics was then placed on the agar with the help of sterile forceps and pressed gently.
7. Within 30 minutes of applying the discs, the plate was inverted and then it was incubated at 37℃ for 16-18 hours.
8. After proper incubation, the diameter of zone of inhibition was measured and result was interpreted based on CLSI guideline.

APPENDIX IV

Nepal Standard of DFTOC

०१:०१ दुध दुध भन्नाले स्वस्य गाई वा भैसीको थुनबाट दुहेको रर स्वभाविक अवस्थाको स्वच्छ ताजा श्रावलाई सम्झनुपर्छ ।

०१.०२ गाईको दुध (Cow milk): गाईको दुध भन्नाले ३.५ प्रतिशत दुधको चिल्लो (Milk fat) र सो चिल्लो बाहेक दुधको ठोस पदार्थ ९:षपि कयष्मि लयत ाबत० ७.५ प्रतिशत भन्दा घटि नएको दुध सम्झनु पर्छ ।

०१.०५ प्रशोधित दुध (Processed Milk): प्रशोधित दुध भन्नाले आंशिक रुपमा दूधको चिल्लो झिकी वा नझिकी वा दूधको चिल्लो रहित दुध ठोस पर्दाथ घोली वा नघोली दूधको चिललो ३.० प्रतिश दूधको चिल्लो वाहेक दुध ठोस पदार्थ ८.० प्रतिशतमा घटी नभएको तथा निरोगन प्रक्रिया (Pasteurization) वा जिवाणु हनन प्रक्रिया (Sterilization) सम्पन्न गरी तयार गरिएको तरल दूधलाई सम्झनुपर्छ । यसमा निरोगन प्रक्रिया गरिएको प्रशोधित दूधलाई प्रशोधित तथा पास्चुराइज्ड दुध र जिवाणु हनन प्रक्रिया सम्पन्न प्रशोधित दुधलाई प्रशोधित तथा स्टरिलाइज्ड दुध भन्न वा लेख्न सकिनेछ ।

द्रष्टव्य: उपर्युक्त सव्केत नम्बर ०१.०५ मा उल्लिखित दूध वा क्रिमको निरोगन प्रक्रिया (Pasteurization) गर्दा दूध वा क्रिमलाई कम्तीमा ६३ सेण्टिग्रेडमा ३० मिनेटको समयभन्दा कम नहुने गरी सम्पूर्ण दूध वा क्रिमलाई तताई वा अन्य कुनै बढी तापक्रममा निरोगन क्रिया पुग्ने समयसम्म राखी त्यसपछि तुरुन्त सम्पूर्ण दूध वा क्रिमलाई १० सेन्टिग्रेड वा त्यो भन्दा कम तापक्रम सम्म चिस्याईएको हुनुपर्नेछ र देहाय मापदण्ड बमोजिम निरोगन प्रक्रिया पूरा गेको हुनुपर्नेछ ।

१. फोस्फाटेज टेस्ट नेगेटिभ हुनुपर्नेछ ।

२. निरोगन गरिएको दूध तथा अन्य दुग्ध तरल पर्दाथ विक्रिमा राखिएको भए वा तयार गरिएको भएमा प्रति मिलितिटरमा कोलीफर्म (Coliform) गणना शुन्य हुनुपर्नेछ ।

Indian Standards

1. Pasteurized Milk

The bacterial criteria prescribed by the Bureau of Indian Standards (BIS 1992) stipulated that the plate count of pasteurized milk, at the plant in the final container, should not exceed 30,000 per mL and the coliform should be absent in 1:10 dilution of pasteurized milk.

2. Raw milk

The Bureau of Indian Standard (BIS 1992) prescribed the following criteria as a guideline for grading of milk based on total viable count.

Grade	Total Viable Count (Lakh/ mL)
Very good	Less than 2 Lakh
Good	2-10 Lakh
Fair	10-50 Lakh
Poor	More than 50 Lakh

Coliform should be absent in 1: 100 dilutions of satisfactory grade raw milk

Detection of milk quality qualitatively by MBRT based on DFTQC guidelines:

Milk sample	MBRT (Minutes)	Quality of milk	Approx. No. of Bacteria
Cultured	0-30	Poor	>20000000
Raw	31-120	Fair	>4000000
Heated	121-180	Good	>500000
Pasteurized	181-480	Excellent	<500000

Composition of Milk Obtained from Different Animals:

S. No.	**Animals**	**Water (%)**	**Protein (%)**	**Fat (%)**	**Lactose (%)**	**Ash (%)**
1	Cow	87.29	3.42	3.66	4.92	0.71
2	Buffalo	83.20	4.50	6.50	5.00	0.80
3	Human	87.60	1.20	3.80	7.00	0.21
4	Camel	87.67	3.45	3.02	5.15	0.71
5	Cat	83.05	7.00	4.50	4.85	0.60
6	Dog	74.55	3.15	10.20	11.30	0.80
7	Yak	82.6	5.4	6.5	4.6	0.9
8	Goat	86.5	3.5	4.5	4.7	0.8

APPENDIX V

BIOCHEMICAL CHARACTERISTICS OF COLIFORM ISOLATES.

Tests	Results
Catalase	Positive
Oxidase	Negative
Oxidative-fermentative	Fermentative
Motility	Positive
Indole	Positive
MR	Positive
VP	Negative
Citrate utilization	Negative
TSI	Yellow/ Yellow, H_2S^-, Gas^+
Urease	Negative

Biochemical properties of *Escherichia coli*

Tests	Results
Catalase	Positive
Oxidase	Negative
Oxidative-fermentative	Fermentative
Motility	Negative
Indole	Negative
MR	Negative
VP	Positive
Citrate utilization	Positive
TSI	Yellow/ Yellow, H_2S^-, Gas^+
Urease	Positive

Biochemical properties of *Klebsiella* spp.

Tests	Results
Catalase	Positive
Oxidase	Negative
Oxidative-fermentative	Fermentative
Motility	Positive
Indole	Negative
MR	Positive
VP	Negative
Citrate utilization	Positive
TSI	Yellow/ Yellow, H_2S^+, Gas^+
Urease	Variable

Biochemical properties of *Citrobacter* spp.