ANTIBIOTIC SUSCEPTIBILITYTESTING OF ISOLATED COLIFORMS FROM PANEER MARKETED 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

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RECOMMENDATION

This is to recommend that **Ms. Manisha Pradhan,** Symbol No.500330042, T.U. Registration No. 5-2-33-189-2017, has carried out project work entitled, **"Antibiotic susceptibility testing of isolated coliforms from paneer marketed in Kathmandu"** for the requirement to project work in Bachelor of Science (B.Sc.) degree in Microbiology under our supervision in the department of Microbiology, Amrit campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U), Nepal.

To my knowledge, this wok has not been submitted for any other degree. She 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.

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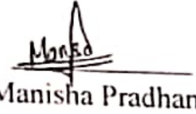
DECLARATION

This project work entitled **"Antibiotic susceptibility testing of isolated coliforms from paneer marketed 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 Mr. Atmaz Kumar Shrestha and co-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.



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[Date: 21, July, 2022]

On the recommendation of **Mr. Atmaz Kumar Shrestha** and **Asst. Prof. Suchitra Thapa,** this project work is submitted by **Manisha Pradhan,** Symbol No. 500330042 T.U. Registration No 5-2-33-189-2017 entitled **"Antibiotic susceptibility testing of isolated coliforms from paneer marketed in Kathmandu"** is forwarded by the Department of Microbiology, Amrit Campus, for the approval to the Evaluation Committee, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

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This project work (PRO-406) entitled **"Antibiotic susceptibility testing of isolated coliforms from paneer marketed in Kathmandu"** by Manisha Pradhan Symbol No 500330042 and T.U. Registration No 5-2-33-189-2017 under the supervision of Atmaz Kumar Shrestha and co-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 the Bachelor of Science (B.Sc.) degree in Microbiology report has been accepted and forwarded to the Controller of Examination, Institute of Science and Technology, Tribhuvan University, Nepal for the legal procedure.

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ABSTRACT

Paneer is a valuable dairy product due to its high nutritional value, distinctive texture, and flavor makes it a good meat substitute. Poor hygiene during preparation, storage and handling of foods including dairy products can lead to microbial contamination in developing nations like Nepal. The aim of this study was to screen microbial quality of paneer and to determine the antibiotic susceptibility testing of total coliforms and thermotolerant coliforms. Here, 30 paneer samples were randomly collected from dairy shops and other different locations in Kathmandu and samples were processed at Department of Microbiology, Amrit Campus during April to June 2022. Serial dilution of sample was performed and by using pour plate method, total coliform count and thermotolerant coliform count was determined. All isolated organisms were identified by various biochemical tests and antibiotic susceptibility pattern of coliforms was carried out by Kirby-Bauer disc diffusion method. In this study, 16.6% of paneer samples were within requirements of DFTQC and FSSAI with maximum count of 4.5×10^5 cfu/gm and thermotolerant bacteria with maximum count of 9.6×10^4 cfu/gm. Coliforms isolated from paneer were *Escherichia coli* (46.9%), *Klebsiella* spp. (37.5%) and *Citrobacter* spp. (15.6%). Thermotolerant coliforms were *Escherichia coli* (58.5%), *Klebsiella oxytoca* (11.8%), *Klebsiella* spp. (17.6%) and *Citrobacter* spp. (11.8%). All isolates of total coliforms (n=32) and thermotolerant coliforms (n=17) showed 100% sensitivity towards Gentamycin and Cotrimoxazole. Resistance towards Ampicillin (40%), Cefoxitin (30%) and Azithromycin (30%) were shown by thermotolerant *E. coli*. Resistance towards Cefoxitin (66.7%), Azithromycin (33.3%) were shown by thermotolerant *Klebsiella* spp. The highest resistance for *E. coli* was found in Azithromycin (40%) and Ampicillin (40%) in total coliforms and thermotolerant coliforms. However, no multiple- antibiotic resistant was found in isolates of total and thermotolerant coliforms. The majority of the paneer samples in this investigation did not meet the microbiological requirements. Sitapaila provided the greatest number of microbiologically acceptable samples, whereas Naikap provided the least number. Thus, it is necessary to monitor and control the quality of paneer sold in Kathmandu.

Key words: Antibiotic susceptibility test, coliforms, multiple-antibiotic resistant, thermotolerant coliforms, Paneer.

शोधसार

पनिर एक महत्वपूर्ण डेरी उत्पादन हो किनभने यसको उच्च पोषण मूल्य, विशिष्ट बनावट, र स्वादले यसलाई मासुको राम्रो विकल्प बनाउँछ। दुग्धजन्य पदार्थ लगायतका खानेकुराको तयारी, भण्डारण र ह्यान्डलिङको समयमा कमजोर सरसफाइले नेपालजस्ता विकासोन्मुख राष्ट्रहरूमा खानेकुरा ब्याक्टेरियाबाट प्रदुषित हुन्छ। यस अध्ययनको उद्देश्य पनीरको माइक्रोबियल गुणस्तर जाँच्नु र टोटल कोलिफर्म र थर्मोटोलरेन्ट कोलिफर्महरूको एन्टिबायोटिकको संवेदनशीलता परीक्षण गर्नु थियो। यहाँ २०२२ को अप्रिलदेखि जुन महिनासम्म काठमाडौंका डेरी पसल र अन्य विभिन्न स्थानबाट ३० वटा पनिरको नमूना संकलन गरिएको थियो। पहिलो नमूनाको सेरिअल दिलुशन गरियो र पोउर प्लेट विधि प्रयोग गरेर, कोलिफर्म गणना र थर्मोटोलेरेन्ट कोलिफर्महरूको गणना गरिएको थियो। सबै पृथक नमुना विभिन्न बायोकेमिकल परीक्षणहरूद्वारा पहिचान गरिएको थियो र कोलिफर्महरूको एन्टिबायोटिक संवेदनशीलता किर्बी-बाउर डिस्क डिफुज्न विधिद्वारा परीक्षण गरिएको थियो। यस अध्ययनमा १६.६% पनिर नमूनाहरू DFTQC र FSSAI को मापदण्डहरू भित्र थिए र टोटल कोलिफम ब्याक्टेरियाको अधिकतम गणना ४.५×१०^{५} cfu/gm र थर्मोटोलेरेन्ट ब्याक्टेरियाको अधिकतम गणना ९.६×१०^{४} cfu/gm थियो। पनिरबाट नमुना कोलिफर्महरू *Escherichia coli* (४६.९%), *Klebsiella* spp. (३७.५%) र *Citrobacter* spp. (१५.६%) थिए। थर्मोटोलेरेन्ट कोलिफमहरू *Escherichia coli* (५८.५%), *Klebsiella oxytoca* (११.८%), *Klebsiella* spp. (१७.६%) र *Citrobacter* spp. (११.८%) थिए। कुल कोलिफर्महरू (n=३२) र थर्मोटोलरेन्ट कोलिफर्महरू (n=१७) को सबै आइसोलेट्सले कोट्रिमोक्साजोल र जेन्ताम्यिसन तर्फ १००% संवेदनशीलता देखायो। थर्मोटोलरेन्ट *E.coli* लाई एम्पिसिलिन (४०%), सेफोक्सिटिन (३०%) र एजिथ्रोमाइसिन (३०%) मा रेसिस्तान्स देखाइएको थियो। सेफोक्सिटिन (६६.७%), र एजिथ्रोमाइसिन (३३.३%) को रेसिस्तान्स थर्मोटोलरेन्ट *Klebsiella* spp. द्वारा देखाइएको थियो। सबैभन्दा बढी रेसिस्तान्स *E. coli* को एजिथ्रोमाइसिन (४०%) र एम्पिसिलिन (४०%) कुल कोलिफर्म र थर्मोटोलरेन्ट कोलिफर्ममा पाइयो। तथापि, कुल र थर्मोटोलरेन्ट कोलिफर्म्सको पृथक ठाउँमा कुनै पनि मल्टिपल-एन्टिबायोटिक रेसिस्तान्स पत्ता लागेको थिएन। यस अध्ययनमा प्रयोग गरिएको बजारबाट लिइएको पनीरको नमूनाहरूको धैरै मात्रामा माइक्रोबायोलोजिकल मापदण्डहरू पूरा नभएको पाइयो। सितापाइलाबाट माइक्रोबायोलोजिकल रूपमा स्वीकार्य नमूनाहरूको अधिकतम संख्या र नैकापबाट सबैभन्दा कम स्वीकार्य नमूनाहरू प्राप्त गरिएको थियो। त्यसैले काठमाडौँमा बजारीकरण हुने पनिरको गुणस्तर अनुगमन, नियन्त्रण र जोखिम मूल्याङ्कन गर्न आवश्यक छ।

Key words: Antibiotic susceptibility test, coliforms, multiple-antibiotic resistant, thermotolerant coliforms, Paneer.

LIST OF ACRONYMS AND ABBREVIATIONS

ATCC	American Type Culture Collection
APHA	American Public Health Association
AST	Antibiotic Susceptibility Test
BAM	Bacteriological Analytical Manual
BIS	Bureau of Indian Standards
CASA	Commercial Agriculture for small holders and Agro business
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
DFTQC	Department of Food Technology and Quality Control
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agricultural Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FDA	Food and Drug Administration
FSSAI	Food Safety and Standard Authority of India
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Point
ICAR	Indian Council of Agricultural Research
ISO	International Standard Organization
MA	MacConkey Agar
mcg	Microgram
MDR	Mutiple Drug Resistant
MHA	Muller Hinton Agar
MOALD	Ministry of Agriculture and Livestock Development
NCRP	National Cattle Research Program
NIFTEM	National Institute of Food Technology Entrepreneurship and Management Ministry of Food Processing Industry
VRBA	Violet Red Bile Salt Agar

LIST OF SYMBOLS

ºC	Degree celsius
+	Positive
-	Negative
±	Equal or less
≤	Less than
≥	Greater than
%	Percentage

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CHAPTER 1

1. INTRODUCTION

1.1 General introduction

Paneer is a non-fermented cheese made from fresh milk, where the milk is coagulated with an acidic agent (like lemon juice, vinegar, or curd) (Deshmukh and Vyas, 2017). It has a fairly high level of fat (22–25%), protein (16–18%) and a low level of lactose (2.0–2.7%). It is also a good source of fat soluble vitamins A and D (Kumar, *et al.*, 2014). Paneer is characterized by a marble white color, mild acidic flavor with slightly nutty taste, spongy body, cohesive and compact texture (Desai, 2007, Khan and Pal, 2011). In recent years, paneer has gained global popularity as a rich source of animal protein for vegans and vegetarians (Khan and Pal, 2011; Zimmerman, 2017). Nutritional value of paneer is high and it is also recommended for patients of diabetes, coronary heart disease, dental caries and pregnant women (Chopra and Mamtani, 1995). Paneer has a short life span of about 2-3 days at refrigeration storage without much deterioration in the quality, but freshness of the product is lost after 1 day (Dhankhar, 2014) and according to information given in packaging labelled in vacuum sealed DDC manufactured Paneer, the shelf life of paneer is 3 months. In developing countries, poor hygiene during preparation, storage and handling of foods including dairy products can lead to microbiological contamination.

The coliforms and *Escherichia coli* are used as indicators of poor microbiological quality of food particularly fecal contamination. The contamination of paneer by pathogenic bacteria can cause gastrointestinal illnesses in the consumers (Pal, *et al.,* 2018). Therefore, food-borne illnesses are major international health problems and important causes of reduced economic growth (WHO, 2002). It is reported that an estimated 600 million (almost 1 in 10 people in the world) fall ill after eating contaminated food and 420 000 die every year, resulting in the loss of 33 million healthy life years (DALYs) (WHO, 2015). Hence, it is important that the milk and milk products intended for human use must be free from pathogens, such *as Campylobacter jejuni, Escherichia coli* O157H:7*, Listeria monocyogenes, Salmonella, Staphylococcus aureus* and *Yersinia enterocolitica,* which produce serious disease in the consumers (Pal, *et al.,* 2018*).* Although these pathogens usually cause self-limiting gastroenteritis, invasive diseases and complications also may occur. Similarly, systemic Salmonellosis infections can be life- threatening, and Shiga toxin producing *E. coli*, particularly *E. coli* 0157:H7, can

cause bloody diarrhea and hemolytic uremic syndrome (Griffin, 1995). It is important that foodborne illness outbreaks are investigated timely and proper environmental assessments are done so that appropriate prevention strategies are identified (Lynch, Tauxe and Hedberg, 2009). According to CDC, the etiology of majority (68%) of reported foodborne-illness outbreaks is unknown. Analysis of foodborne outbreak data helps in the estimation of the proportion of human cases of specific enteric diseases attributable to a specific food item (Greig and Ravel, 2009). Food unsafe for consumption causes 600 million cases of foodborne diseases and 420,000 deaths every year, and 56 million people die each year (Ritchie and Roser, 2018; WHO, 2015). This data indicates that 7.69% (600 million) individuals of world populations (7.8 million) suffer from foodborne diseases every year and 7.5% (4,20,000 death) of all deaths (56 million) annually are due to foodborne illnesses.

The development of antibiotic resistance by microorganisms has been linked to the use and misuse of antibiotics for therapeutic purposes and for growth promotion (Forshell and Wierup, 2006; Adzitey, 2015). Antibiotic resistance is a global problem, but demand for antibiotics continues to rise, particularly to treat patients suffering from bacterial infections (Liu, *et al.,* 2015). Antibiotic use is the key factor in the selection of resistant bacteria, with community and hospital settings forming the principal ecological niches of emergence in human health (WHO, 2015). The emergence of antibiotic resistance along the food chain is thus a major global public health issue with several studies having reported food animals and products being colonized or infected and contaminated by antibiotic resistant strains such extended spectrum-beta-lactamase (ESBL) producing Enterobacteriaceae (viz. *Salmonella spp., Shigella spp., Escherichia coli., Klebsiella spp., etc.* (Fisher, *et al*., 2012; Al Bayssari, *et al*., 2015). Antibiotic resistant bacteria may reach humans i) indirectly along the food chain through consumption of contaminated food or food derived products, and ii) following direct contact with colonized/ infected animals or biological substances such as blood, urine, feces, saliva and semen among others (Chang, *et al.,* 2015). The food borne pathogens that are able to survive and thrive in post pasteurization environments can lead to recontamination of dairy products. These pathways pose a risk to consumers (Oliver, 2005). So, the presence of knowledge about the current scenario about the antibiotic resistance of different microbes isolated from milk and dairy products are less in number.

The composition of many milk products makes them good media for the outgrowth of pathogenic microorganisms. Potential also exists for the contamination of milk with residues of veterinary drugs, pesticides and other chemical contaminants. In many countries milk and milk products form a large portion of the diet of consumers especially infants, children, and pregnant and lactating women. Therefore, implementation of the proper hygienic control of milk and milk products throughout the food chain is essential to ensure the safety and suitability of these foods for their intended use (FAO, 2004). Thus, establishment of national standard microbial guideline of coliforms for paneer and continuous microbial quality monitoring is essential.

1.2 Rationale

This study is essential because consumers of dairy products need to be aware of the many foodborne diseases due to contaminated milk products. This study deals with presence of indicator bacteria such as coliforms contributes the understanding of hygiene status increasing consumer knowledge of the danger of foodborne illness caused by milk products such as paneer. Food poisoning and diarrhea could result from consuming paneer that has poor microbiological quality. Monitoring dairy products frequently has a big impact on their quality. The study will help to find out where paneer of inferior quality is sold and determine antibiotic susceptibility of coliforms.

1.3 Objectives

1.3.1 General objectives

- To isolate and enumerate coliform bacteria from paneer sample and perform antibiotic susceptibility testing of bacteria isolated from paneer marketed in Kathmandu.

1.3.2 Specific objectives

- To isolate enumerate total coliforms bacteria and thermotolerant coliform bacteria from paneer samples.
- To identify coliforms from paneer samples.
- To determine antibiotic susceptibility pattern of coliforms isolated from paneer samples.

CHAPTER 2

2. LITERATURE REVIEW

Paneer is a rich source of animal protein available at a comparatively lower cost and forms an important source of animal protein for vegetarians (Shrivastava and Goyal, 2007).

2.1 History of paneer and manufacturing process

Paneer is indigenous to South Asia and was first introduced in India by Afghan and Iranian travellers. Earlier milk was coagulated using heat and sour milk or by proteolytic enzymes from creeper like Putika or bark of Palasa (*Butea frondosa*), Kuyala or Jujuka (Jujube) (Chopra and Mamtani, 1995). Cheese manufactured using high heat and acid precipitation without resorting to use of starter culture (similar to Indian paneer) was practiced in many countries of South Asia and Central South and Latin America. People during the Kusana and Saka Satavahana periods (AD 75-300) used to consume a solid mass, whose description seems to the earliest reference to the present day paneer (Mathur, *et al.,* 1986; Mathur, 1991). The solid mass was obtained by mixing heated milk with curd. The credit of developing paneer is usually given to nomadic people from west Asia (Mathur, *et al.,* 1986).

Paneer manufacturing process: The manufacture of paneer involves standardization of milk, heat treatment, coagulation, draining of whey, pressing, dipping in chilled water and packaging.

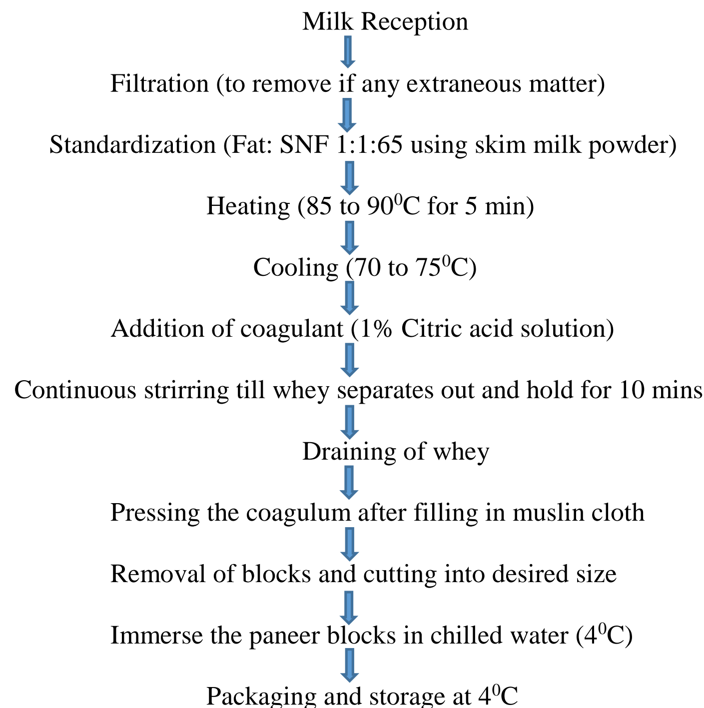


Figure 1: Flowchart of manufacturing of paneer (NIFTEM, 2020)

2.2 Production of milk and milk products in Nepal

At present the dairy sector of Nepal is contributing 0.247% percent of the world's total milk production. Nepal's milk output is estimated to be 2.05 million metric tons (FAOSTAT, 2019). The per capita availability of milk has also increased to a level of about 158.9 gram per day (RAN, 2015). This sector has its importance in reducing poverty through creating employment and income generating opportunities that is ensured through regular cash flow from urban to the rural areas (Neupane, *et al.*, 2018). The population growth rate of Nepal is found to be 1.35 percent per annum. Under this assumption, the current milk production should be raised by 4 percent so as to meet the WHO recommended minimum value of 250 gm per day per-capita milk consumption by the year 2025. In contrast, current milk production growth stands only at 3.09 percent per annum (Upadhyay, 2017). At present, of the milk product sold in the formal sector, fluid milk has the biggest share (36.9%) of the value of an average retailer's dairy transactions. Ghee (20.6%) and yoghurt (14.9%) are the other major chunks in the dairy produce market, followed by paneer (7.4%) and ice cream (6.4%) (CASA, 2020). In Nepal milk producing farmers are suffering from the problem of milk holidays during flush season and the self-life of raw and pasteurized milk is very low as compared to different milk products. So, to overcome the problem of milk holiday there should be diversification of milk products which increase the storage time leading to more benefits to farmers. Due to the change in feeding habits, people prefer milk products better than the whole milk. On the other hand, the cost of production and selling prize of raw milk is similar. So, farmers are not getting better return from milk selling. Product diversification may be one of the best practices to get more benefits to milk producers (MoALD, 2075).

2.3 Factors affecting quality of dairy products

Microorganisms have an absolute demand of water. Each microorganism has maximal, optimal and minimal water activity (a_w) for the growth. This range depends on factors like solute, nutritional value for culture medium, temperature, oxygen supply, pH, inhibitors, etc. The water activity (a_w) necessary for survival of coliforms is 0.98 (Frazier, 2007).

2.4 Epidemiology of foodborne disease outbreaks due to dairy products

More than 150 million people fall sick 175,000 die every year after consuming contaminated and unsafe food in the World Health Organization South East Asia Region

including Nepal (WHO, 2015). *E. coli* O157:H7 was first identified as a pathogen in 1982 in an outbreak of bloody diarrhea traced to hamburgers from a fast-food chain (Riley, *et al.*,1983); it was subsequently shown to have a reservoir in healthy cattle (Martin, *et al.,* 1986). Along with new pathogens, an array of new food vehicles of transmission has been implicated in recent years. *E. coli* 0157:H7 has caused illness through Diarrhea is the most common symptoms of food borne illnesses, other serious consequences include kidney and liver failure, brain and neutral disorders, and death (Binali, *et al.,* 2006). *E. coli* O157:H7 has caused illness through an ever-broadening spectrum of foods, beyond the beef and raw milk that are directly related to the bovine reservoir. In 1992, an outbreak caused by apple cider showed that this organism could be transmitted through a food with a pH level of less than 4.0, possibly after contact of fresh produce with manure (Besser, *et al.,* 1993). Food poisoning from Enteropathogenic *Escherichia coli* (EPEC) often results in similar health outcomes as that of Salmonellosis, which can be acquired from ingesting 10^6 organisms (Todar, 2008). Some virulent *E. coli* can even lead diarrheal diseases, gastroenteritis (Barrett, *et al.*, 2005). The public health hazard of *E. coli* organism has been emphasized by several investigator as they have been implicated in human cases of gastroenteritis, epidemic diarrhea in infants, sporadic diarrhea in children as well as food poisoning (Cohen and Kerdahi, 1996 and Veronozy, 1997).

2.5 Coliforms in food and dairy products

In microbiological testing, an indicator organism is defined as marker that reflects the general microbiological condition of a food or environment (Chapin, *et al.,* 2014). Since 1914, the United States has used coliform organisms to indicate the microbiological quality and safety of drinking water (USDT, 1914). Milk and dairy products are rich sources of many nutrients and hence, they serve an excellent medium for microbial growth (Ledenbach and Marshall, 2009). It is stated that cleaning and sanitation of milk and milk products contact surfaces contribute around 60% of the total contamination in a dairy plant (Pal and Mahendra, 2015). Food poisoning by the coliform group occurs due to the consumption of food, water contaminated with coliforms. They are common cause of diarrhoeal illness globally, and are the most common cause of urinary tract infection (Poolman and Wacker, 2016). *E. coli* is frequently associated with travellor's diarrhea. Two toxins types are produced: heat stable toxins (ST), which can withstand heating at 100^0C for 15 min and are acid resistant, and the heat- labile toxins (LT) which are inactivated at 60^0C for 30 min and at low pH. Faecal contamination of water supplies

and contaminated food handlers have been most frequently implicated in outbreaks caused by EPEC, EIEC, ETEC (Adams and Moss, 2004). According to WHO-2015, the number of coliform present in any food or food product should be 0 cfu/gm. Similar, standard value/guideline is given by Department of Food Technology and Quality Control in Nepal for the quality assurance (DFTQC, 2010).

2.6 Morphology and virulence factors of coliforms

Coliforms are a group of gram-negative, non-spore-forming, rod-shaped aerobes and facultative anaerobes that inhabit the intestinal tract of all vertebrates. They can ferment lactose when incubated at 35–37^{0}C, producing acid and gas (Feng, *et al*., 2020). Thermotolerant coliform bacteria are the coliform organisms that are able to ferment lactose at 44–45^{0}C; the group includes the genus *Escherichia* and some species of *Klebsiella*, *Enterobacter*, and *Citrobacter* (WHO, 2017). *E. coli* is a gram negative bacteria that may be straight, rod shaped and arranged singly or in pairs. *Escherichia coli* is one of the predominant enteric species in the human gut and, as part of the normal intestinal flora (FDA, 2012). Currently there are six recognized groups: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Nataro, 1998). *E. coli* is often used as a marker organism. Recovery of *E. coli* is used as a reliable indicator of fecal contamination and indicates the possible presence of enteropathogenic microorganisms which constitute a public health hazard (Bali, *et al*., 2013). *Klebsiella* species are ubiquitous in nature and commonly are in water and food. *Klebsiella* spp. is a Gram negative, cylindrical rod shaped, non-motile, lactose fermenting and facultative anaerobic bacteria measuring 2 μm by 5 μm. The factors that are implicated in the virulence of *Klebsiella* spp. include capsular polysaccharides, lipopolysaccharides, fimbrial adhesins, and siderophores. *Enterobacter* spp. are facultatively anaerobic, gram negative bacilli, 0.6-1 μm in diameter and 1.2-3 μm long, motile by means of peritrichous flagella and have class 1 fimbriae. *Enterobacter* spp. strains produce many potential virulence factors, including enterotoxins like haemolysin and cytotoxins. *Citrobacter freundii* is another opportunistic pathogen, but also is a resident of the human gastrointestinal tract. It is gram negative, rod shaped, non sporing (Chakraborty, 2019, Cheesebrough, 2006 and Forbes, *et al*., 2007).

2.7 Prevalence of coliforms in paneer

The following list of published literatures is comprised of works by various scholars who have studied the microbiological quality of paneer:

Singh and Singh, (2000) studied the market samples of paneer collected from Agra city and found coliform count (3.05 log10 cfu/g) for laboratory made sample.

Goyal, *et al.* (2007) studied that on quality evaluation of market paneer from India and found that coliform count (1.1×10^{4}cfu/gm).

Dhole, *et al.* (2009) evaluated 70 samples of fresh paneer from seven vendors of Ahmednagar City for microbiological quality. The average coliform count in the market sample of paneer were ranged from 12.6 x 103 to 23.2 x 103 cfu/g with an average of 17.543 x 103 cfu/g. Out of total paneer samples (70) 97.2 per cent samples showed significantly higher coliform counts per gram when compared with that of BIS standards.

Acharya, *et al.* (2012) studied milk samples from Kathmandu valley and found that coliforms were present in 65.1% of total milk samples. The mean value of Total Coliform Count (TCC) of pasteurized, unpasteurized and raw milk was 2.9×10^{4}cfu/mL, 6.3×10^{5}cfu/mL and 1.6×10^{5}cfu/mL respectively. *E. coli* was isolated from 18.75% of the pasteurized milk samples, 20% from 25 raw milk samples and 40% from 25 unpasteurized milk samples.

Dabholkar, *et al.* (2013) studied paneer of 32 samples from Nagpur city and had bacteriological counts ranging from 1×10^{6} to 8.2×10^{7}cfu/gm in which *E. coli* was found 72% contaminated of the samples.

Poudel, *et al.* (2015) studied milk samples from Kathmandu Nepal and found that coliform bacteria within the range of 0.9×102 cfu/mL to 4.27×102 cfu/mL.

Chauhan, *et al.* (2016) studied paneer samples from Allahabad, India and found that coliforms was absent in all samples. So, the product was found to be good and proper hygienic condition were maintained during the preparation, handling and storage.

Dabir, *et al.* (2017) studied Physico-chemical, sensory, textural and microbiological quality of 80 packets of 8 different brands of paneer marketed in Bengaluru city and found that coliform count was 3.95 to 7.65×10^{1}/gm and about 75% of brands did not conform to FSSAI labelling requirements.

Girdharwal, (2018) studied 80 samples of Indian paneer from Delhi India and found that coliform count between 3.95-7.65$\times10^{1}$cfu/gm.

Gogoi, *et al*. (2018) studied bacteriological assessment of paneer of 30 samples from Assam India and found that positive samples of *E. coli* was 20(66.7%) and 80% of paneer samples of coliform count was above the acceptable standards.

Kandil, *et al*. (2018) studied the marketed dairy samples collected from Mansoura city, Egypt found that *E. coli* were observed in 86% in cheese samples.

Dongare, (2019) studied quality evaluation of market paneer from Prabhani Maharastra, India market. In this study no coliforms were detected in all of the analyzed samples.

Simkhada, *et al.* (2019) studied 64 paneer samples collected from Kathmandu valley and found that maximum number of total coliform count was found in Lazimpat i.e, 2.0$\times10^{5}$ cfu/gm with an average count of 3.1$\times10^{4}$ cfu/gm and in Thamel with maximum count of 4.9$\times10^{3}$ cfu/gm and 57.8% samples were found to be contaminated with coliforms and 42.2% of sample within guideline. Among the 52 isolates from total coliform, *E. coli* was detected in 37.7% of paneer sample, *Klebsiella* spp. (35.5%), *Klebsiella oxytoca* (25%) and *Citrobacter* species (8%). From 24 isolates of thermotolerant coliforms, *E. coli* was detected in 54.2% of paneer sample, *Klebsiella* spp. (29.2%) and *Klebsiella oxytoca* (16.7%).

Anandh, *et al*. (2021) studied traditional styled paneer pickles samples from India in which no coliform count was observed in ready to eat paneer pickle.

2.8 Antibiotic Resistance

Antibiotics are chemical substance derivable from microorganism that kills or inhibits microorganism and cures infection. Antibiotics are frequently used in veterinary practices to treat & prevent animal disease. They are also used at sub-therapeutic levels to increase feed efficiency, promote growth and prevent diseases (Mosalagae, *et al*., 2011). In dairy animal commonly used antimicrobials are Sulphonamides, Aminoglycosides, Tetracyclines, Macrolids, Quinolones, β- lactams etc. Currently, approximately 80% of all food-producing animals receive medication for part or most their lives (Lee, *et al*., 2001). Antibiotic resistance is a global health crisis, decreasing the efficacy of antibiotics. More than 2.8 million antibiotic- resistant infections occur in the U.S each year. More than 35,000 people die as a result, Antibiotic Resistant (AR) Threats Report (CDC, 2019). Antimicrobial resistance in bacterial pathogens is a challenge that is associated with high

morbidity and mortality. Multidrug resistance patterns in Gram- positive and -negative bacteria are difficult to treat and may even be untreatable with conventional antibiotics (Frieri, *et al.,*2017).

2.8.1 Antibiotic susceptibility pattern of *E. coli*

Vural, *et al*. (2009) studied that antibiotic sensitivity tests from Turkey and found that all *E. coli* strains were sensitive to Amikacin, Cefoxitin, Gentamicin, Levofloxacin, etc. 23.1% of *E. coli* was detected to be resistant to Ampicillin, Cotrimoxazole (28.9%) respectively.

Gautam, *et al*. (2015) studied paneer samples (n=75) in which 38 (50.6%) isolates and *E. coli* was present in 22 (57.8%). *E. coli* showed sensitive result for Gentamycin, Ciprofloxacin and Ofloxacin, intermediate result for Tetracycline and Kanamycin and also found to be resistant against the Amoxcycillin. The present result showed that the *E. coli* were most sensitive against ciprofloxacin with the zone of inhibition of 26mm.

Acharya, *et al*. (2017) studied milk samples from Kathmandu valley and found that all the 18 isolated *E. coli* were sensitive to all the administered antibiotics disc Amikacin, Gentamicin, Chloramphenicol, Cotrimoxazole, Levofloxacin except ampicillins. Among 18 *E. coli* isolates, 16.7% were susceptible and remaining 83.3% were resistant to the antibiotic ampicillins.

Tark, *et al*. (2017) determined that *E. coli* strains isolated from raw milk samples harbored the high prevalence of resistance against Tetracycline (23.3%), Ampicillin (16.6%), and Cotrimoxaazole (11.2%).

Gogoi, *et al*. (2018) studied bacteriological assessment of paneer sold in Assam, India and antibiotic susceptibility pattern and found that 20 isolates of *E. coli* was 100% sensitive to Gentamycin, 100% Chloramphenicol, 75% Ampicillin. Cefoxitin showed 100% resistant.

Simkhada, *et al. (*2019) studied paneer samples that the antibiotic susceptibility pattern from Kathmandu valley and found that the resistance to Ampicillin was detected in *Escherichia coli* (82.4%), isolates of *Escherichia coli* were also resistance to Tetracycline (35.5%). Besides these, 100% sensitivity was seen towards Ceftriaxone, Chloramphenicol, Ciprofloxacin, Cotrimoxazole, Ofloxacin and Amikacin by all three thermotolerant coliform isolates.

Rai, *et al*. (2020) reported that antibiogram of *Escherichia coli* isolated from milk sold in Kathmandu district and found that all the isolates showed 100% susceptibility towards Chloramphenicol while none of the isolates showed susceptibility towards Ampicillin. Also, the susceptibility for Tetracycline and Nalidixic acid were 81.2%and 78.1% respectively.

Ghimire, *et al*. (2020) studied that antibiogram profiling and thermal inactivation of *E. coli* from milk samples from Dharan, Nepal and found that all the *E. coli* isolates (6) were resistant to Ampicillin and least resistant to Cefoxitin. Chloramphenicol, Amikacin, Azithromycin, and Nalidixic acid were found highly effective to *E. coli*.

Hammad, *et al*. (2022) studied cheese samples from Egypt and analysis of the susceptibility status of thermotolerant coliforms isolates revealed that (42.8%, 60/140) isolates were susceptible to all antibiotics tested. The highest rate of resistance was observed for Ampicillin (30.7%, 43/140), Cefixime (16.4%, 23/140), Streptomycin (16.4%, 23/140), Tetracycline (12.8%, 18/140).

2.9 Methods of Quality Evaluation of Paneer

Sensory Evaluation: The products developed are subjected to sensory characteristics comprising of color, flavor, texture, taste and overall acceptability of paneer samples by a panel of five judges. The evaluation of the product was carried out by using the 9-point Hedonic scale (Srilakshmi, 2002). The data obtained was analyzed statistically for its validity by using factorial design and critical difference (C.D.) technique (Imran and Coover, 1983).

Chemical analysis: Fat, Protein, Lactose, Ash, Total Solids and pH are estimated by using standard procedure laid down in FSSAI Lab Manual in Milk and Milk Products (FSSAI, 2015).

Microbial Analysis: Standard Plate count, Yeast & Mold count, and Coliform test are evaluated by using standard procedure laid down by regulatory agencies (Indian Standard, 1977; Manual in Dairy Bacteriology, 1972; Indian Standard, 1999; FSSAI Manual of methods of Analysis of Foods, 2012). Microbiological criteria as per 10th amendment regulations, 2016, Food Safety and Standards Authority of India (FSSAI) has specified microbiological requirement for process hygiene and food safety.

Microbiological requirements for Paneer

Microbes	Minimum	Maximum
Aerobic Plate Count	1.5×10^5/gm	3.5×10^5/gm
Coliform (cfu/gm)	10/gm	100/gm
Yeast and mould (cfu/gm)	50/g	150/gm
E.coli	Less than 10/gm	Less than 10/gm
S. aureus	10/gm	100/gm

Source: Paneer Standard, FSSAI, 2012.

The FSSAI regulations specify a permissible standard limit of microorganisms for Paneer are mentioned above. These are indicative contamination values above which corrective actions are required in order to maintain the hygiene of the Paneer production process in compliance with the law.

According to document mentioned in Nepal Rajpatra about the quality of Paneer "Paneer refers to a solid made from the milk of a healthy cow or buffalo or both by completing the pasteurization process and souring milk with lactic acid or citric acid. It should not be foul-smelling or moldy and should not be mixed with artificial colors and other substances and should have the following quality parameter".

S.No	Description	Standard
1	Moisture	Not more than 70%
2	Milk fat	Not less than 50%

Source: Food Standard, DFTQC, 2019.

There should be absence of coliform in dairy products according to Nepal standard (coliform count should be zero) (DFTQC, 2010).

2.9.1 Coliform count

Microbiological count methods are the accepted laboratory methods used to estimate the microbial population of a tested substance. This method is used by the dairy industry for estimating the microbial populations in most types of dairy products and samples and for determining quality and sources of contamination at successive stages of processing (Laird, *et al.* 2004). Enumeration of coliforms present in products intended for human consumption or feeding of animals, by means of the technique of counting colonies on a solid medium, after incubation at 37^0C. Preparation of poured plates, using a solid selective culture medium, and using a specified quantity of the test sample if the initial product is liquid, or using a specified quantity of an initial suspension in the case of other products. Preparation of other pairs of poured plates, under the same conditions, using

decimal dilutions of the test sample or of the initial suspension (BIS, 2002). Violet Red Bile Agar (VRBA) is recommended for selective isolation, detection and enumeration of coli-aerogenes bacteria in water, milk other dairy food products (APHA,1978 and Salfinger, *et al*., 2015). Selectivity of VRBA can be increased by incubation under anaerobic conditions and or at elevated temperature, i.e. equal to or above 42^0C (HiMedia, 2022).

2.9.2 Antibiotic susceptibility testing

The performance of antimicrobial susceptibility testing (AST) of bacterial pathogens is an important task to determine susceptibility to the antibiotics. Laboratory antimicrobial susceptibility testing can be performed using dilution technique and disc diffusion technique.

Dilution Susceptibility Tests: Dilution technique measures the minimium inhibitory concentration (MIC). They can be used to measure the minimum bactericidal concentration (MBC) which is the lowest concentration of antimicrobial required to kill bacteria (Cheesbrough, 2006).

Disc Diffusion Susceptibility Tests: This tests are used by most laboratories to test routinely for antimicrobial susceptibility. A disc of blotting paper is impregnated with a known volume and appropriate concentration of an antimicrobial, and is placed on a plate of susceptibility testing agar uniformly inoculated with test organism. The antimicrobial diffuses from the disk into the medium and the growth of test organism is inhibited at a distance from the disc that is related to the susceptibility of the organism (Cheesbrough, 2006).

CHAPTER 3

3. MATERIALS AND METHODOLOGY

3.1 Materials

All the materials including equipment, glassware, chemicals, reagents and microbiological media used are listed in Appendix I.

3.2 Methods

3.2.1 Study site/ Sampling site

This study was conducted in Kathmandu district that lies in Province 3 of Nepal. Sampling and analysis of paneer samples were performed during April to June 2022. It is most densely populated district of Nepal with population of 20,17,532 in 2022 (CBS,2022). In this study, samples were collected from the five different locations i.e Naikap, Kalanki, Bafal, Sitapaila and Thamel city of Kathmandu district.

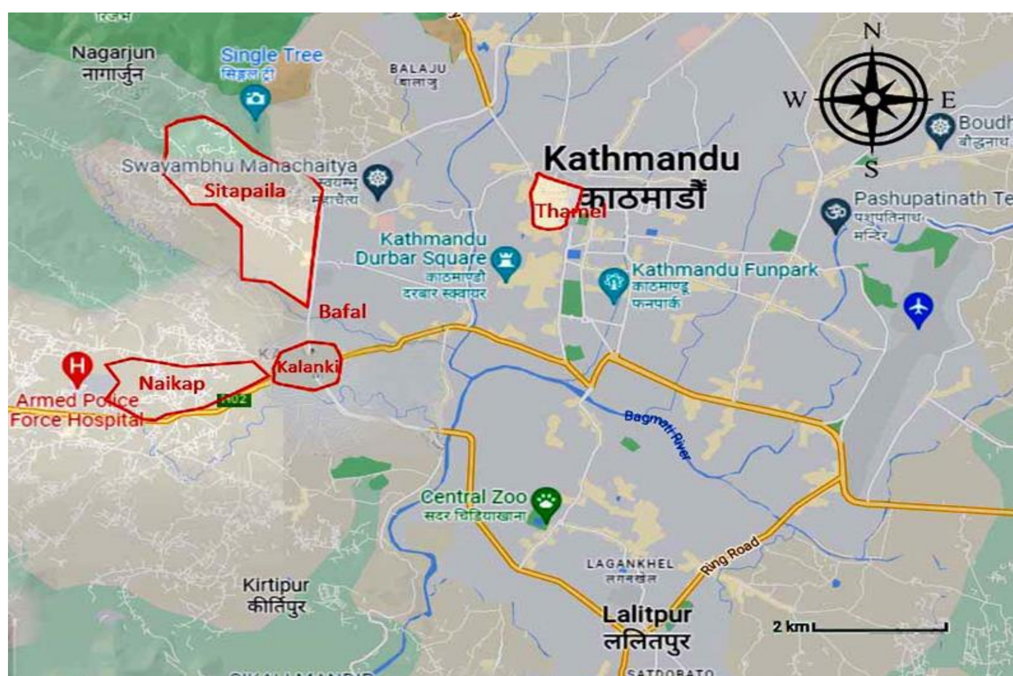


Figure 2: Map showing different sampling localities (Naikap, Kalanki, Bafal, Sitapaila and Thamel)

3.2.2 Study design

The method of study was cross- sectional. Sampling and laboratory analysis of paneer samples for isolation, enumeration, identification and antibiotic susceptibility testing of coliforms and thermotolerant coliforms were performed.

3.2.3 Sample source, sample size and sampling method

Convenient sampling was followed for the sample collection. A total of 30 paneer samples (6 from each location from different dairy shops of Naikap, Kalanki, Bafal, Sitapaila and Thamel) were collected from Kathmandu district.

3.2.4 Sample collection and transportation

The paneer samples were collected from different dairy shops from selected sampling sites in a sterilized container and condition as provided by the vendor. Paneer samples were transported in pre sterile foils and an ice cold box and processed in the laboratory within 2 hours of sample collection (Haddad and Yamani, 2017).

3.3 Laboratory analysis and processing

All the laboratory analysis of paneer samples were done in microbiology laboratory of Department of Microbiology, Amrit Campus, Tribhuvan University. Bacterial quality of paneer sample was determined by FSSAI specification (FSSAI, 2012).

3.3.1 Preparation and enumeration of sample

Paneer sample (10gm) was homogenized aseptically in 90mL of 0.85% sterile normal saline in a conical flask to obtain 1 in 10 dilutions (10^{-1}) and mixed using vortex mixture. The 10-fold serial dilutions were prepared from the initial dilution using sterile normal saline (0.85% NaCl) for which 1 mL from the 10^{-1} dilution was transferred to 10^{-2} labeled test tube containing 9 ml sterile normal saline and similarly diluted upto 10^{-6}. Each dilution 1 mL of diluted sample was transferred into sterile petriplate aseptically. Then, around 10 mL molten violet red bile salt agar (VRBA) was poured in each petriplate containing 1ml diluted sample and swirled into clockwise and anti-clockwise directions and allowed to solidify. Then, again molten VRBA was added to solidified VRBA for double layering and incubated at 37^0C and 44^0C for 24-48 hrs. After that, enumeration of isolated colonies i.e coliforms (pinkish red colonies) in paneer sample were calculated by using colony forming unit (cfu)/gm formulae. Samples were examined according to APHA, 1978; BAM, 2002; BIS, 2002; Cheesbrough, 2007; Forbes, *et al*., 2007; Girdharwal, 2018; Haddad and Yamani, 2017; Harrigan and McCance, 1976; Himedia, 2022 and Powers, *et al*., 1978) with slight modification.

$$\text{cfu/gm} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Weight of sample taken}}$$

3.3.2 Isolation and identification of coliforms

Isolation and identification of total coliform was done according to BAM online (2001) and Haddad and Yamani (2017). From each sample, 1-2 pinkish red colonies on Violet Red Bile agar were subjected to identification of total coliforms and thermotolerant coliforms. An isolated pinkish red colony on VRBA was transferred to Nutrient Broth and incubated at 37°C for 4 hours and then sub-cultured on MacConkey agar (MA) and incubated at 37°C for 24 hours. From this pure culture, colony morphology was studied and gram staining and biochemical tests such as Catalase test, Oxidase test, Oxidative-Fermentative test, Sulphide Indole Motility test, Methyl Red test, Voges-Proskauer test, Citrate Utilization test, Triple Sugar Iron test and Urease test were done for identification of total coliforms and thermotolerant coliforms (Cheesbrough, 2006 and Forbes, *et al*., 2007).

3.3.3 Antibiotic susceptibility testing

Antibiotic susceptibility testing of isolated coliforms and thermotolerant coliforms was done following the Kirby-Bauer disk diffusion method on Muller Hinton Agar (MHA) according to clinical and laboratory standards institute. The antimicrobial agents tested against Coliforms and thermotolerant coliforms were Ampicillin (10mcg), Chloramphenicol (30mcg), Nalidixic acid (30mcg), Cotrimoxazole (25mcg), Tetracycline (30mcg), Cefoxitin (30mcg), Levofloxacin (5mcg), Amikacin (30mcg), Azithromycin (15mcg) and Gentamycin (10mcg). The zone of diameter was measured in millimeter scale. The zone of diameter for individual antimicrobial agents was then interpreted into sensitive, intermediate and resistant categories according to recommended guideline for common pathogen (CLSI, 2018).

3.4 Quality Control

All the necessary quality controls of media and culture plates were performed during the experiment. The media lot no. and expiry date were also checked. *Escherichia coli* (ATCC, 25922) was taken as control.

3.5 Data analysis

Data was collected in laboratory by experimental method. These collected data were tabulated in observational table and entered in MS Excel data sheet and analyzed by interpreting the outcome of result. Primary data were collected from experiment and secondary data were collected from published books and articles.

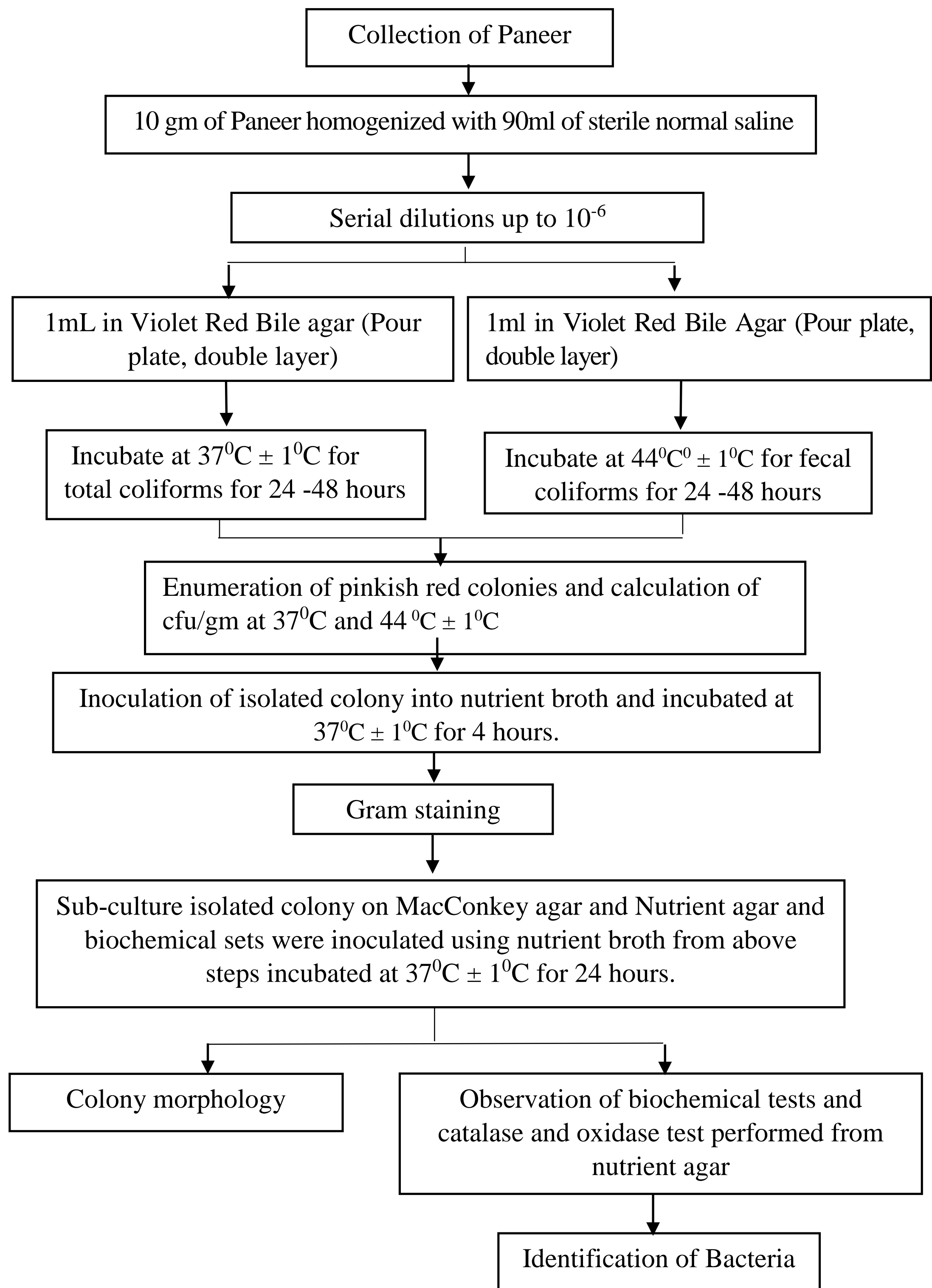


Figure 3: Flowchart for enumeration and identification of coliforms from paneer (APHA, 1978; BAM, 2002; BIS, 2002; Cheesbrough, 2007; Forbes, *et al*., 2007; Girdharwal, 2018; Haddad and Yamani, 2017; Harrigan and McCance, 1976; Himedia, 2022 and Powers, *et al*., 1978).

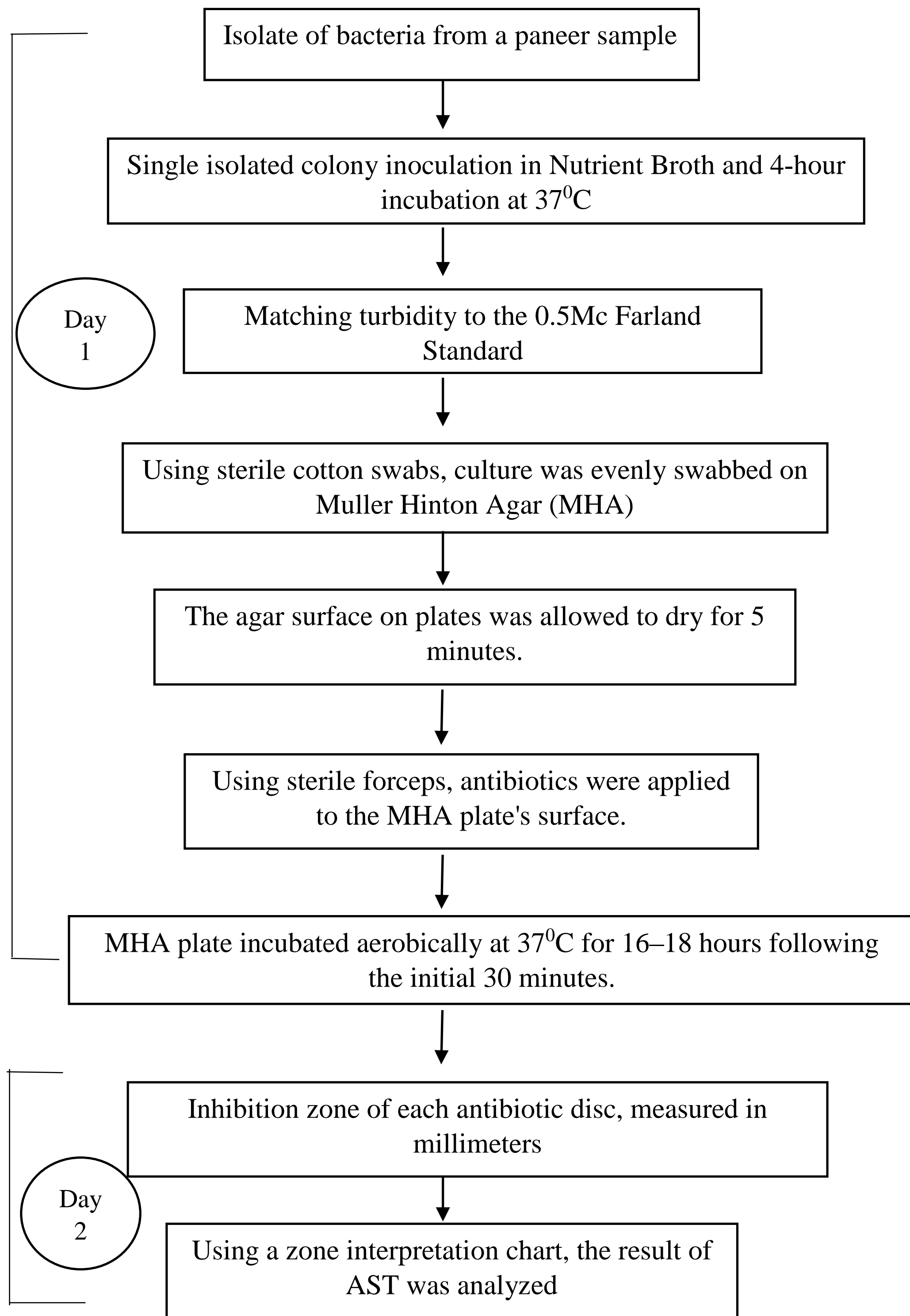


Figure 4: Flowchart for assessing the antibiotic susceptibility testing of coliforms isolated from paneer (CLSI 2018).

CHAPTER 4

4.1 Result

A total of 30 paneer samples from five different localities of Kathmandu district Naikap, Kalanki, Bafal, Sitapaila and Thamel were assessed for detection of total coliforms, thermotolerant coliforms and their antibiotic susceptibility pattern was determined.

4.1.1 Coliforms present in paneer samples

Out of 30 paneer samples, 25 (83.3%) samples coliforms were detected and shown in (Table 1). The maximum number of coliforms were present in samples from Naikap (6) followed by Kalanki (6), Bafal (5), Thamel (5) and Sitapaila (3) respectively.

Table 1: Location wise distribution of coliforms growth at 37^{0}C

Location	Sample size (n)	Total coliforms Detected sample	
		Number	%
Naikap	6	6	100
Kalanki	6	6	100
Bafal	6	5	83.3
Sitapaila	6	3	50
Thamel	6	5	83.3
Total number of sample (N)	**30**	**25**	**83.3%**

4.1.2 Total coliform count

Total coliform count from paneer samples from different localities were represented on Table 2. The highest average coliform count 1×10^5 cfu/gm were found in paneer sample from Naikap followed by Kalanki, Bafal, Thamel and Sitapaila respectively.

Table 2: Location wise distribution of total coliform count

Location	Sample size(n)	Minimum count	Maximum count	Average cfu/gm
Naikap	6	1	4.5×10^5	1×10^5
Kalanki	6	-	2.7×10^5	6.7×10^4
Bafal	6	-	2.1×10^5	6.5×10^4
Sitapaila	6	-	1×10^5	1.6×10^4
Thamel	6	-	1.7×10^5	5.6×10^4
Total sample (N)	**30**			

- not detected

4.1.3 Quality of paneer according to DFTQC specification for total coliform count

Maximum number of microbiologically acceptable samples were obtained from Sitapaila and 3 (50%) were acceptable. The acceptable samples in Thamel and Bafal were 1 (17%) respectively. The samples from Naikap and Kalanki samples were not within DFTQC specification.

Table 3: Quality of paneer according to DFTQC specification

Location	Size (n)	Sample within the DFTQC specification		Sample exceeding the DFTQC specification	
		0 cfu/gm		>0 cfu/gm	
		Number	%	Number	%
Naikap	6	-	-	6	100
Kalanki	6	-	-	6	100
Bafal	6	1	17	5	83
Sitapaila	6	3	50	3	50
Thamel	6	1	17	5	83
Total number of sample (N)	**30**	**5**	**16.6**	**25**	**83.4**

4.1.4 Quality of paneer according to FSSAI specification for total coliform count

Maximum number of microbiologically acceptable samples were obtained from Sitapaila and 3 (50%) were acceptable. The acceptable samples in Thamel and Bafal were 1 (17%) respectively. The samples from Kalanki and Naikap were microbiologically unsatisfactory according to FSSAI specification.

Table 4: Quality of paneer according to FSSAI specification

Location	Size (n)	Sample within the FSSAI specification		Sample exceeding the FSSAI specification	
		<10 cfu/gm		>10 cfu/gm	
		Number	%	Number	%
Naikap	6	-	-	6	100
Kalanki	6	-	-	6	100
Bafal	6	1	17	5	83
Sitapaila	6	3	50	3	50
Thamel	6	1	17	5	83
Total number of sample (N)	**30**	**5**	**16.6**	**25**	**83.4**

4.1.5 Thermotolerant coliforms present in paneer samples

Out of 30 paneer samples, 16 (53.3%) samples thermotolerant coliforms were detected and shown in (Table 5). The maximum number of contaminated samples was from Naikpap (5), Kalanki (4), Bafal (3), Sitapaila and Thamel (2) respectively.

Table 5: Location wise distribution of thermotolerant coliforms growth at 44^0C

Location	Sample size (n)	Thermotolerant coliforms Detected sample	
		Number	%
Naikap	6	5	83.3
Kalanki	6	4	66.6
Bafal	6	4	66.6
Sitapaila	6	2	33.3
Thamel	6	2	33.3
Total number of sample (N)	**30**	**17**	**56.7%**

4.1.6 Thermotolerant coliform count

Thermotolerant coliform count from paneer samples from different localities were represented on Table 6. The highest average coliform count 2×10^4 cfu/gm were found in paneer sample from Naikap followed by Kalanki, Bafal, Thamel and Sitapaila respectively.

Table 6: Location wise distribution of thermotolerant coliform count

Location	Sample size(n)	Minimum count	Maximum count	Average cfu/gm
Naikap	6	-	9.6×10^4	2×10^4
Kalanki	6	-	5.7×10^4	1×10^4
Bafal	6	-	1.5×10^4	2.6×10^3
Sitapaila	6	-	1×10^3	1.6×10^2
Thamel	6	-	1.2×10^4	2×10^3
Total sample (N)	**30**			

- not detected

4.1.7 Total coliforms and thermotolerant coliforms from paneer samples

Out of 30 samples, total of 32 isolates of total coliforms and 17 isolates of thermotolerant coliforms were processed during the study. Total coliforms isolated from paneer samples were *Escherichia coli* (46.9%), *Klebsiella* spp. (37.5%), *Citrobacter* spp. (15.6%) and thermotolerant coliforms isolated from paneer samples were *E. coli* (58.5), *Klebsiella* spp. (17.6%), *Klebsiella oxytoca* (11.8%) and *Citrobacter* spp. (11.8%) as shown in Figure 5.

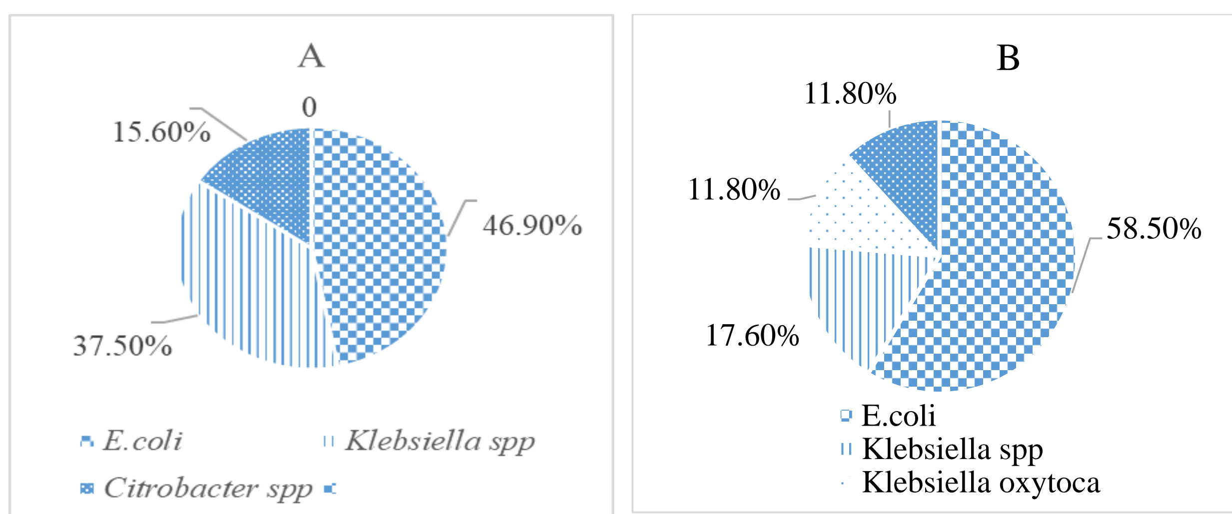


Figure 5: Percentage of identified isolates of coliforms (A) and thermotolerant coliforms (B)

4.1.8 Antibiotic susceptibility pattern of total coliforms

The pattern of antibiotic susceptibility of total coliforms is represented in Table 7 below. The findings demonstrated that isolates of *E. coli, Klebsiella* spp. and *Citrobacter* spp. were 100% sensitive to Chloramphenicol. The result revealed that 26.7% of *E. coli, Klebsiella* spp. 50% and *Citrobacter* spp. 100% were resistant to Ampicillin. However, no multiple-antibiotic resistant was found in isolates of total coliforms.

Table 7: Antibiotic susceptibility pattern of total coliforms for *E. coli*, *Klebsiella* spp. and *Citrobacter* spp.

Antibiotics	***E. coli*** N=15			***Klebsiella* spp.** N=12			***Citrobacter* spp.** N=5		
	S(%)	**I(%)**	**R(%)**	**S(%)**	**I(%)**	**R(%)**	**S(%)**	**I(%)**	**R(%)**
Ampicillin (10 mcg)	10(66.6)	1(6.7)	4(26.7)	6(50)	0	6(50)	0	0	5(100)
Chloramphenicol (30mcg)	15(100)	0	0	12(100)	0	0	5(100)	0	0
Nalidixic acid (30 mcg)	14(93.3)	1(6.7)	0	12(100)	0	0	4(80)	1(20)	0
Cotrimoxazole (25 mcg)	15(100)	0	0	12(100)	0	0	5(100)	0	0
Tetracycline (30 mcg)	15(100)	0	0	10(83.4)	2(16.6)	0	5(100)	0	0
Cefoxitin (30 mcg)	7(46.6)	4(26.8)	4(26.6)	7(58.3)	0	5(41.7)	3(60)	0	2(40)
Levofloxacin(5mcg)	13(86.6)	1(6.7)	1(6.7)	12(100)	0	0	4(80)	1(20)	0
Amikacin (30 mcg)	10(66.6)	5(33.4)	0	10(83.4)	2(16.6)	0	5(100)	0	0
Azithromycin (15mcg)	0	9(60)	6(40)	0	9(75)	3(25)	1(20)	2(40)	2(40)
Gentamycin (10mcg)	15(100)	0	0	12(100)	0	0	5(100)	0	0

S= no. of isolates sensitive, R= no. of isolates resistant, N= no. of isolates, I= Intermediate

4.1.9 Antibiotic susceptibility pattern of thermotolerant coliforms

The Thermotolerant coliforms *E. coli* (10), *Klebsiella* spp. (3), *K. oxytoca* (2) and *Citrobacter* spp. (2) were tested for antibiotic susceptibility and the results revealed that 40% of *E. coli*, 100% of *Klebsiella* spp., 100% of *K. oxytoca* and 100% of *Citrobacter* spp. were resistance to Ampicillin. *E. coli* also showed 30% resistance towards Azithromycin and Cefoxitin respectively. In addition to that, complete sensitivity was found in Nalidixic acid, Cotrimoxazole, Gentamycin respectively.

However, no multiple-antibiotic resistant was also found in this isolates of thermotolerant coliforms.

Table 8: Antibiotic susceptibility pattern of thermotolerant coliforms for *E. coli.*

Antibiotics	***E. coli*** **N =10**		
	S (%)	**I(%)**	**R (%)**
Ampicillin (10 mcg)	5(50)	1(10)	4(40)
Chloramphenicol (30mcg)	10(100)	0	0
Nalidixic acid (30 mcg)	10(100)	0	0
Cotrimoxazole (25 mcg)	10(100)	0	0
Tetracycline (30 mcg)	8(80)	2(20)	0
Cefoxitin (30 mcg)	4(40)	3(30)	3(30)
Levofloxacin(5mcg)	9(90)	1(10)	0
Amikacin (30 mcg)	7(70)	3(30)	0
Azithromycin (15mcg)	3(30)	4(40)	3(30)
Gentamycin (10mcg)	10(100)	0	0

S= no. of isolates sensitive, R= no. of isolates resistant, N= no. of isolates, I= Intermediate

Table 9: Antibiotic susceptibility pattern of thermotolerant coliforms for *Citrobacter* spp.

Antibiotics	***Citrobacter* spp. N=2**		
	S (%)	**I(%)**	**R (%)**
Ampicillin (10 mcg)	0	0	2(100)
Chloramphenicol (30 mcg)	2(100)	0	0
Nalidixic acid (30 mcg)	2(100)	0	0
Cotrimoxazole (25 mcg)	2(100)	0	0
Tetracycline (30 mcg)	2(100)	0	0
Cefoxitin (30 mcg)	0	0	2(100)
Levofloxacin(5mcg)	1(50)	1(50)	0
Amikacin (30 mcg)	1(50)	1(50)	0
Azithromycin (15mcg)	0	1(50)	1(50)
Gentamycin (10mcg)	2(100)	0	0

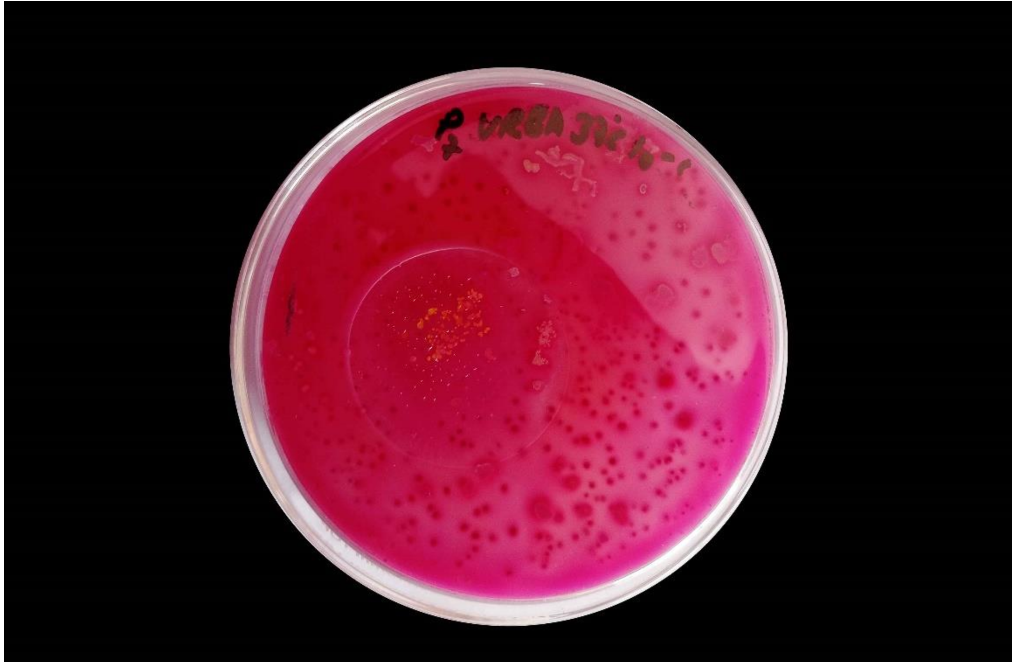
S= no. of isolates sensitive, R= no. of isolates resistant, N= no. of isolates, I= Intermediate

Table 10: Antibiotic susceptibility pattern of thermotolerant coliforms for *Klebsiella* spp. and *Klebsiella oxytoca*

Antibiotics	***Klebsiella* spp. N =3**			***Klebsiella oxytoxa* N=2**		
	S (%)	**I(%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Ampicillin (10 mcg)	0	0	3(100)	0	0	2(100)
Chloramphenicol (30mcg)	3(100)	0	0	1(50)	0	1(50)
Nalidixic acid (30 mcg)	3(100)	0	0	2(100)	0	0
Cotrimoxazole (25 mcg)	3(100)	0	0	2(100)	0	0
Tetracycline (30 mcg)	1(33.3)	2(66.7)	0	2(100)	0	0
Cefoxitin (30 mcg)	1(33.3)	0	2(66.7)	0	1(50)	1(50)
Levofloxacin(5mcg)	3(100)	0	0	2(100)	0	0
Amikacin (30 mcg)	2(66.7)	1(33.3)	0	2(100)	0	0
Azithromycin (15mcg)	0	2(66.7)	1(33.3)	0	2(100)	0
Gentamycin (10mcg)	3(100)	0	0	2(100)	0	0

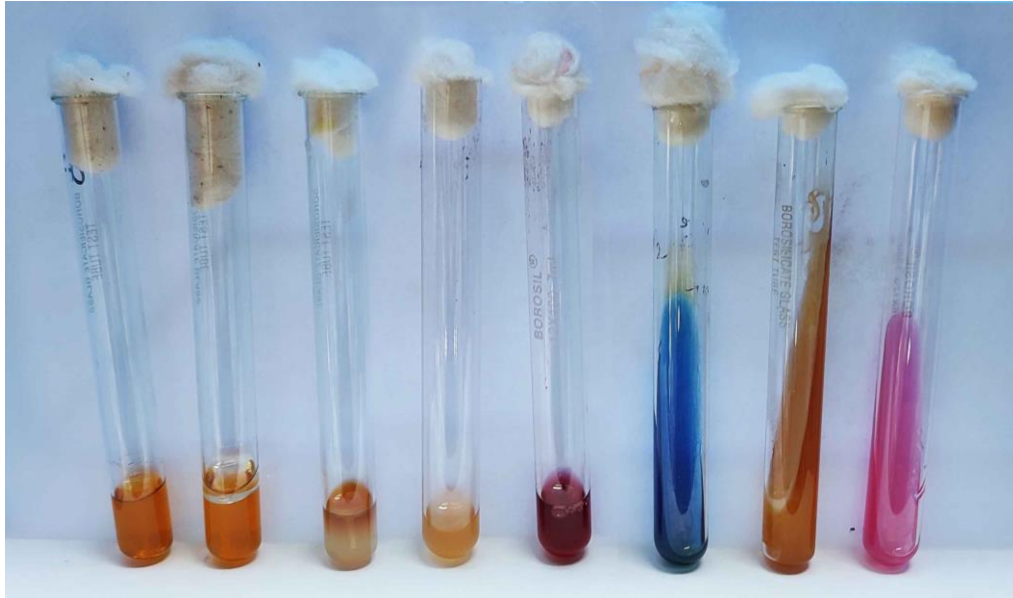
S= no. of isolates sensitive R= no. of isolates resistant, N= no. of isolates, I= Intermediate

PHOTOGRAPHS

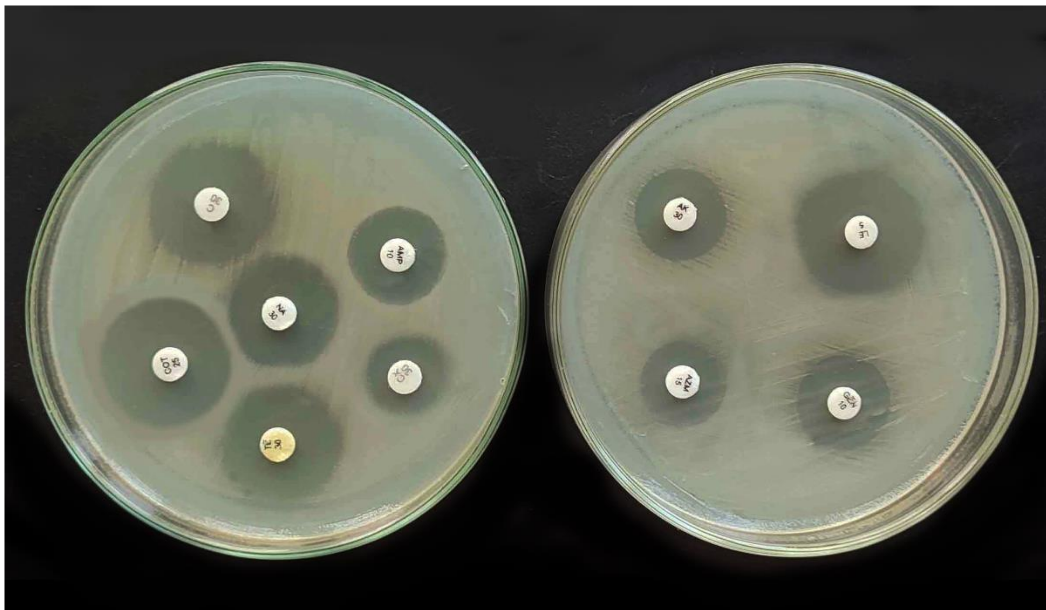


Photograph 1: Colonies of coliforms in VRBA

Photograph 2: *Klebsiella* spp. on MacConkey agar plate



Photograph 3: Biochemical test of *Klebsiella* spp. (OF= Oxidative fermentative, SIM= Sulphide Indole Motility, MR= Methyl Red, VP= Voges Proskauer, TSI= Triple Sugar Iron, C= Citrate, U= Urease from left to right)



A **B**

Photograph 4: Antibiotic susceptibility test of *Escherichia coli*
Figure A: {COT= Cotrimoxazole(25mcg), TE= Tetracycline(30mcg), Cx= Cefoxitin(30mcg) AMP=Ampicillin (10mcg), C= Chloramphenicol(30mcg), NA=Nalidixic Acid (30mcg)
Figure B: {AK= Amikacin (30mcg), AZM= Azithromycin (15 mcg), GEN= Gentamycin (10mcg) and LE= Levofloxacin (5 mcg)}

Photograph 5: Sample processing in laboratory of Amrit Campus

4.2 Discussion

Paneer have been often reported to be contaminated with coliforms and its contamination may be due to various reasons like milking process, transportation, manufacturing, handling, storage, packaging (Nayak *et al.,* 2013).

In the microbial examination of 30 paneer samples, coliforms were present in 83.3% of the samples. Similarly, Simkhada, *et al.* (2012) reported that coliforms were present in 57.8% of the samples. Maximum number of total coliform count was found in Naikap i.e, 4.5×10^5 cfu/gm with an average count of 1×10^5 cfu/gm. Simkhada, *et al.* (2012) also reported that maximum number of total coliform count was found in Lazimpat i.e, 2×10^5 cfu/gm with an average count of 3.1×10^4 cfu/gm which was lower than our study. Dhole, *et al.* (2009) reported that average coliform count were ranged from 12.6 x 10^3 to 23.2 x 10^3 cfu/gm with an average of 17.5 x 10^3 cfu/gm. In our study, the average coliform count was ranged from 5.6×10^4 to 1×10^5 cfu/gm with an average of 6×10^4 cfu/gm that was higher count as compared with Dhole, *et al.* (2009). Similarly, Cisse *et al*., 2019 reported that total coliform densities were 1.98×10^3 CFU/ml which was lower count as compared to our study.

Girdharwal, (2018) found that coliform count between $3.95\text{-}7.65\times10^1$cfu/gm which was lower count as compared to our study. Coliform in food products and *E. coli* can even lead diarrheal diseases, gastroenteritis, food poisoning (Barrett, *et al*., 2005). Goyal *et al*., (2007) reported paneer samples contaminated with coliforms 1.1x10^4 cfu/gm which was lower than result of coliforms in our study. The majority of the contamination is assumed to have occurred during packaging process and also due to the contamination from different source including the poor quality of raw milk and processing in uncontrolled environments (Sameh, 2016 and Shrivastava, 2007). Other sources of these organisms is fecal contamination, suspected to be of human origin. However, all of these pathogens are considered to also be zoonotic (Todar, 2008).

In this study, 83.4 % samples showed significantly higher coliform counts per gram and exceeded the DFTQC and FSSAI requirements. However, coliform count was higher than the reported by Simkhada, *et al.* (2012) which was 57.58% but, lower than Dhole, *et al.* (2009) who reported 97.2% samples exceeded requirements of FSSAI. In similar study carried out by Dabir, *et al.* (2017), 75% of brands did not conform to FSSAI requirements of coliforms which was lower than our study. Presence of coliform (either total coliforms

or thermotolerant coliform or both) implies that paneer samples were unfit for consumption. Locally manufactured Paneer is inherently susceptible to contamination with various microorganisms because it is always stored in the open while being sold. Therefore, incorrect handling and cleanliness could result in contamination, which would impact on consumers' health. If presence of coliforms in food is neglected, it can cause a serious health hazards (Girdharwal 2018). Coliform in food may results in gastrointestinal illness as severe cholera-like syndrome, gastroenteritis, epidemic diarrhoea and food poisoning (Ekici, *et al.* 2018).

In our study, thermotolerant coliforms were present in 56.7% in the total samples and maximum number of thermotolerant coliform count was found in Naikap i.e., 9.6×10^4 cfu/gm with an average count of 2×10^4 cfu/gm. Similarly, Poudel *et al.*, 2015 reported that coliform bacteria was found within the range of 0.9×10^2 cfu/mL to 4.27×10^2 cfu/mL which was lower count as compared to our study. In case of Paneer manufacturing blocks of paneer are immersed in chilled water (NIFTEM 2020), if the water is contaminated with the thermotolerant coliforms then paneer would also be contaminated. This may be the reason for reporting of high number of thermotolerant coliforms in Paneer samples.

From total coliform, out of 32 bacterial isolates 15 (46.9%) of coliform were identified as *E. coli* followed by *Klebsiella* spp. 12 (37.5%), and *Citrobacter* spp. 5 (15.6%). Likewise, from thermotolerant coliform, 17 isolates of coliform bacteria obtained were identified in which 10 (58.5%) were found to be *E. coli, Klebsiella* spp. 3 (17.6%) *Klebsiella oxytoca* 2 (11.8%) and *Citrobacter* spp. 2 (11.8%). *E. coli* was also found to be most predominant microorganism in studies done by Simkhada, *et al.* (2017) and NCRP, (2019). The high number of coliforms bacteria in Paneer samples in our study might be due to lack of packaging in sales point which leads to easier contamination by handlers.

Similar study was conducted by many researchers and presence of coliform bacteria from paneer was reported. Simkhada, (2019) reported that *Escherichia coli* (32.7%), *Klebsiella* spp. (35%), *Klebsiella oxytoca* (25%), *Enterobacter* spp. (8%) from paneer samples. Similarly, thermotolerant coliforms isolated from paneer were *E. coli* (58.8%), *Klebsiella* spp. (17.6%), *K. oxytoca* (11.8%) and *Citrobacter* spp. (11.8%). Mikcha *et al.*, (2010) reported that *E. coli* (77.1%), *Klebsiella oxytoca* (4.9%) and *Citrobacter freundii* (4.9%) were isolated from pasteurized cow milk samples in Brazil.

Antimicrobial susceptibility testing was done to detect possible drug resistance in the isolated bacteria and to assure susceptibility to drug of choice for particular infections caused by them. Antimicrobial resistance may arise either spontaneously or by selective pressure or due to antimicrobial misuse in humans or animals (Schroder*, et al.,* 2016). In this study, antibiotic susceptibility testing of total coliforms, comprising *Escherichia coli, Klebsiella* spp. and *Citrobacter* spp. showed 100% sensitive towards Chloramphenicol, Cotrimoxazole and Gentamycin respectively. The resistance to Ampicillin was detected in *Escherichia coli* (26.7%), *Klebsiella* spp. (50%) and *Citrobacter* spp. (100%). The isolates of *Escherichia coli* were also resistance towards Cefoxitin (26.7%), Azithromycin (40%), Levofloxacin (6.7%) and *Klebsiella* spp. were resistance towards Cefoxitin (41.7%), Azithromycin (25%). While, Subba, *et al*. (2020) studied that all the *E. coli* isolates were resistant to Ampicillin and least resistant to Cefoxitin, Chloramphenicol, Amikacin, Azithromycin, and Nalidixic acid were found highly effective to *E. coli* which is quite agreement with our result. Gautam, *et al*. (2015) reported that *E. coli* showed sensitive result for Gentamycin, Ciprofloxacin and Ofloxacin, intermediate result for Tetracycline, and was found to be resistant against the Amoxcycillin. Gogoi, *et al*. (2018) reported that *E. coli* were 100% sensitive towards Gentamycin and Chloramphenicol, 75% towards Ampicillin. The results are similar to our study. However, Gogoi, *et al*. (2018) reported 100% resistance of *E. coli* isolates towards Cefoxitin which is not in agreement with our study.

Research done by Chandrasekaran, *et al*. (2014) found 13.4% of *E. coli* isolates were found to be resistant i.e. resistance to 1 or 2 of antimicrobials and few *E. coli* isolates (13.4%) were multi-drug resistant. However, no multiple-antibiotic resistance was found in our study. Resistant to beta lactam antibiotic might be due high use of beta-lactam antibiotics to treat mastitis in cattle NCRP (2019). Rapidly emerging resistant bacteria threaten the extraordinary health benefits that have been achieved with antibiotics. This crisis is global, reflecting the worldwide overuse of these drugs and lack of development of new antibiotic agents by pharmaceutical companies to address the challenge (Bartlett, *et al.,* 2013).

Antibiotic susceptibility testing of thermotolerant coliforms, comprising *E. coli*, *K. oxytoca*, *Klebsiella* spp. and *Citrobacter* spp. showed 40% of *E. coli*, 100% of *K. oxytoca,*100% of *Klebsiella* spp. and 100% of *Citrobacter* spp. were resistant to Ampicillin in our study. Resistance of 30% were also observed against Cefoxitin and

Azithromycin respectively by thermotolerant *E. coli.* Resistance of 66.7% and 33.3% were also observed against Cefoxitin and Azithromycin by *Klebsiella* spp. respectively. Besides these, 100% sensitivity was seen towards Nalidixic acid, Cotrimoxazole and Gentamycin by three thermotolerant coliform isolates. However, Paneto *et al*., (2016) reported that *E. coli* were resistance to Nalidixic acid (40%), Tetracycline (31%), Ampicillin (29%). Simkhada *et al.,* (2019) reported that 23.1% of *E. coli*, 75% of *K. oxytoca* and 57.1% of *Klebsiella* spp. showed resistance to Ampicillin. Resistance of 7.7% and 14.3% was also observed against Tetracycline and Nalidixic acid by *E. coli* and *Klebsiella* spp. respectively. Besides these, isolates thermotolerant coliforms showed 100% sensitivity towards Ceftriaxone, Chloramphenicol, Ciprofloxacin, Cotrimoxazole, Ofloxacin and Amikacin respectively which was quite similar to our study. According to NCRP (2019) from the research in mastitis disease, *Staphylococcus* & *Escherichia coli* were identified as major mastitis causing pathogen. Antibiogram profile indicated that pathogen mostly resistance to Ampicillin and Amoxyclav. Tetracycline, Gentamicin, Ciprofloxacin, Chloramphenicol and Enrofloxacin are effective antibiotics for treatment of mastitis.

CHAPTER 5

5.CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Paneer is an integral part of diet of a significant population of Nepal. Based on the results of coliform count and antibiotic susceptibility testing of paneer, it showed that coliforms were present in paneer i.e not safe for consumption. Maximum number of microbiologically acceptable samples were obtained from Sitapaila and most of unacceptable samples were obtained from Naikap. These findings reflected poor quality of paneer that might be due to unhygienic condition during production, storage and handling of paneer as well also indicated faecal contamination of paneer. Thus, presence of thermotolerant coliforms in paneer marked in Kathmandu poses a health risk to the consumers. Coliforms and thermotolerant coliforms isolated from paneer were susceptible to most of the antibiotics tested in the study. Good hygienic practices from production to market level are required to reduce the microbial load or contamination of paneer in Kathmandu. In this study, resistant to beta lactam antibiotic such as Ampicillin and Cefoxitin was observed. However, no multiple- antibiotic resistance was found in this study. The results of this study suggest that microbial antibiotic susceptibility pattern is crucial for observing the development of antibiotic resistance. The bulk of the samples of paneer from the market that were used in this study did not meet the microbiological standards.

5.2 Novelty and National Prosperity aspect of Project work

Nepal is an agricultural nation with a particular emphasis on the dairy subsector. As paneer is popular dairy products and consumed by all age groups. The available data are low in microbial quality of paneer samples. So, this study is an novel approach and helps to detect total coliform and thermotolerant coliforms bacteria and antibiotic susceptibility testing in paneer samples, the findings have revealed the present status of paneer sold in Kathmandu. At the same time, there are negative effects due to dairy borne disease should be mitigated. Milk production in Nepal not same all year round but the demand for milk products in the market is also have fluctuations. Therefore, routine analysis of these products by the production site must be assessed and antibiotic susceptibility testing of this project can be used in suggesting the effectiveness of the respective drugs which help in providing the proper treatment to the patients. This will help in improving the public health aspect at the

national level. Thus, this study will be useful to understand the quality of paneer in Kathmandu.

5.3 Limitations of the Study

This project was done at a laboratory on the Amrit Campus with a small sample size, and limited funding. Since moisture, pH, water activity, and storage conditions all affect the growth of bacteria in paneer, we were unable to explore the origins of contamination or other factors that may have an impact.

5.4 Recommendations for further work

1. Investigation should be done for other zoonotic pathogens in paneer.
2. Hygiene and Sanitation of paneer should be maintained in production, storage and marketing of paneer.
3. Regular large scale microbial quality monitoring of marketed paneer should be carried out for quality control, to ensure food safety and to prevent foodborne diseases.
4. Therefore, detailed long term study research based on bacteria and along with quantitative analysis of bacterial molecular biology together with preservation techniques in consideration will be necessary in the future.

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APPENDIX I

List of materials

Equipments

1. Autoclave (Life, India)
2. Incubator (Leader,UK)
3. Incubator (Memmert, Germany)
4. Hot air oven (Ambassador)
5. Binocular microscope (COSLAB, India)
6. Refrigerator (LG, India)
7. Electronic weighing balance (Phoenix instrument, Germany)
8. Bunsen burner
9. Ice box (Marina 24S)
10. Thermometers

Glass-wares

1. Beakers
2. Conical flasks
3. Petri dish
4. Pipettes
5. Measuring cylinders

Miscellaneous

1. Aluminium foil
2. Cotton
3. Forceps
4. Pipette filler
5. Labeling tags

Chemicals and reagents used for the identification of organisms

1. Crystal Violet solution
2. Gram's Iodine
3. Acetone - Alcohol
4. Safranine
5. 3% Hydrogen peroxide solution
6. 1% Tetramethyl p–phenyline-diaminedihydrochloride
7. Kovac's reagent

8. MR reagent
9. Barrit's reagent
10. Paraffin oil
11. Normal saline

Microbiological Media (Hi-Media Laboratories Pvt. Ltd.)

1. Nutrient Agar
2. Nutrient Broth
3. Violet Red Bile Agar
4. MacConkey Agar
5. Muller Hinton Agar

Biochemical media

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

Antibiotics used

1. Ampicillin (10mcg)
2. Chloramphenicol (30mcg)
3. Nalidixic acid (30mcg)
4. Cotrimoxazole (25mcg)
5. Tetracycline (30mcg)
6. Cefoxitin (30mcg)
7. Levofloxacin (5mcg)
8. Amikacin (30mcg)
9. Azithromycin (15mcg)
10. Gentamycin (10mcg)

APPENDIX II

Composition and preparation of sample diluent

Normal Saline Ingredients	Gm/litre
Sodium Chloride	8.5 gm
Distilled Water (D/W)	1 litre

Preparation: 8.5 gm of sodium chloride was weighed and transferred to a beaker. Then, around 1000 mL distilled water was added to a beaker and dissolved completely and transferred to 1 litre volumetric flask. Then, distilled water was added upto1 litre mark, and mixed well. Then the solution was dispensed on a test tube and conical flask and then sterilize by autoclaving at 15 lbs pressure for (121^0C) 15 minutes.

APPENDIX III

Procedure for coliform count (pour plate technique)

1. First of all, 10gm of paneer sample was homogenized with 90 mL diluents like normal saline and serial dilutions were made.
2. Samples were serially diluted up to 10^6.
3. The diluted samples are plated using Pour plate technique on violet red bile agar (VRBA), also double layered with VRBA and incubated at 37^0C for 24-48 hours for isolation and enumeration of coliforms.
4. Thermotolerant coliforms are detected by the ability of bacteria to grow at 44 ± 1^0C.
5. Further study of colony morphology and gram staining and biochemical tests are performed for identification of total coliform and thermotolerant coliform.

   (BAM, 2002; BIS, 2002; Cheesbrough, 2007; Forbes, *et al*., 2007; Girdharwal, 2018; Harrigan and McCance, 1976; Haddad and Yamani, 2017; Himedia, 2022 and Powers, *et al*., 1978).

APPENDIX IV

Composition and preparation of different staining reagents

Crystal Violet stain:

Composition	**Gm/litre**
Crystal Violet	20.00gm
Ammonium Oxalate	9.00gm
Ethanol (absolute)	95.00mL
Distilled Water (D/W)	1000mL

Preparation: 20 grams of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 mL of ethanol was added and mixed until the dye dissolved completely. Then, 9 gm of ammonium oxalate was weighed and dissolved in about 200 mL distilled water. Then it was added to the stain. Finally, the volume was made 1 litre by adding distilled water.

Gram's Iodine Solution:

Composition	**Gm/litre**
Potassium Iodide	20gm
Iodide	10gm
Distilled Water (D/W)	1000mL

Preparation: To 1000ml of Distilled water, 20 grams of Potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally, the volume was made 1 litre by adding distilled water.

Acetone-Alcohol Decolourizer:

Composition	**Gm/litre**
Acetone	500mL
Ethanol (absolute)	475mL
Distilled Water (D/W)	25mL

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

Safranin solution:

Composition	**Gm/litre**
Safranin (2.5% solution in 95% ethanol)	100mL
Distilled Water (D/W)	100mL

Preparation:10 grams of safranin was weighed and transferred to a clean bottle. Then, 100mLof distilled water is added to the bottle and mixed well until safranin dissolves completely**.**

APPENDIX V

Composition and preparation of culture media

Violet Red Bile Agar (VRBA) (M049-500G)

Ingredients	**Gm/litre**
Peptone	7.000
Yeast extract	3.000
Lactose	10.000
Bile salts mixture	1.500
Sodium chloride	5.000
Neutral red	0.030
Crystal violet	0.002
Agar	15.000

Final pH (at 25^0C) 7.2 ± 0.2

Preparation: 41.53 grams of VRBA was suspended in 1000 mL purified/distilled water. Then the solution was heated to boiling to dissolve the medium completely. VRBA media was not autoclaved. The media was cool to 45-50^0C and was immediately pour into sterile petri plates containing the inoculum. If desired, the medium can be sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes.

MacConkey Agar (MA)

Ingredients	**Gm/litre**
Peptone	1.50
Casein enzymic hydrolysate	1.50
Gelatin peptone	17.00
Lactose	10.00
Bile salts	1.50
Sodium chloride	5.00
Crystal voilet	0.001
Neutral red	0.03
Agar	15.00

Final pH (at 25^0C) 7.1 ± 0.2

Preparation: 51.53 gm of the MA media was suspended in 1000 mL distilled water and boiled with gentle swirling to dissolve the agar completely, then sterilized by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes. Then the media was cooled to 45-50^{0}C and mixed well and pour into sterile Petri plates. The surface of the medium should be dry when inoculated.

Nutrient Agar (NA)

Ingredients	**Gm/litre**
Peptone	5.00
HM peptone B#	1.50
Yeast Extract	1.50
Sodium chloride	5.00
Agar	15.00

Final pH (at 25^{0}C) 7.4 ± 0.2.

Preparation: 28 grams of the media was suspended in 1000 mL distilled water and boiled to dissolve completely then sterilized by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes. Then, it was cooled to 45-50^{0}C and poured 18-20 mL into sterile petri plates.

Nutrient Broth (NB)

Ingredients	**Gm/litre**
Peptic digest of animal tissue	5.00
Yeast Extract	1.50
Beef Extract	1.50
Sodium chloride	5.00

Final pH (at 25^{0}C) 7.4 ± 0.2

Preparation: 13 gm of NB broth media was suspended in 1000 mL distilled water and boiled to dissolve completely. Then, the media was sterilized by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes.

Muller Hinton Agar (MHA)

Ingredients	Gm/litre
Beef infusion	300.00
Acid hydrolysate of casein	17.50
Starch	1.50
Agar	17.00

Final pH (at 25^0C) 7.3 ± 0.1

Preparation: 38 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely. It was sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. Then the media was cooled to 45-50^0C. Then, it was mixed well and poured into sterile petri plates.

APPENDIX VI

Composition and preparation of biochemical media

(Hugh Leifson's) basal media

Ingredients	**Gm/litre**
Casein enzymic hydrolysate	2.00
Sodium chloride	5.00
Dipotassium Phosphate	0.30
Bromo thymol blue	0.08
Agar	2.00

Final pH (at 25^0C) 6.8 ± 0.2 pen

Preparation (1000mL): 9.38 grams of Hugh Leifson's media was dispensed in distilled water and boiled to dissolve the medium. Then, it was dissed in 100mL amounts completely and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. To first, 100mL of sterile basal medium, 10ml of sterile 10% dextrose solution was added aseptically. To second, 10mL sterile and 10% lactose solution was added in 100mL distilled water. To third, 10mL sterile 10% saccharose solution was added in 100 mL distilled water. It was mixed and then dispensed in 5 ml amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation.

Sulfide indole motility (SIM) Media:

Ingredients	**Gm/litre**
Peptone	30.000
HM Peptone B #	3.000
Peptonized ion	0.200
Sodium Thiosulphate	0.025
Agar	3.000

Final pH (at 25^0C) 7.3 ± 0.2

Preparation (1000mL): 36.23 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely. The media was dispensed in test tubes and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. The tubes were allowed to cool in upright position.

MR-VP broth

Ingredients	Gm/litre
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Agar	2.0

Final pH (at 25^0C) 6.9 ± 0.2

Preparation (1000 mL): 17 gm of the MR-VP media was suspended in 1000 mL distilled water and boiled to dissolve completely. Then the dissolved solution dispensed in test tubes and sterilized by autoclaving at 15 psi pressure (121^0C) for 15 minutes.

Simmon's citrate agar

Ingredients	Gm/litre
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 25^0C) 6.8 ± 0.2

Preparation (1000 mL): 24.2 gm of citrate agar media was suspended in 1000 mL of the distilled water and boiled to dissolve completely. The media was dispensed in test tubes. Then it was sterilized by autoclaving at 15 psi pressure (121^0C) for 15 minutes. Then the solution was cooled down to about 45^0C and allowed to set on slant position.

Triple sugar iron (TSI) Agar

Ingredients	Gm/litre
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0

Dextrose	1.0
Ferrous Sulphate	0.2
Sodium chloride	5.0
SodiumThiosulphate	0.3
Phenol Red	0.024
Agar	12.0

Final pH (at 25^0C) 7.4 ± 0.2

Preparation (1000 mL): 65 gm of the media was suspended in 1000mL distilled water and boiled to dissolve completely. The media was dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. Then the media was allowed to set in slope for obtaining butt and slant.

Urea base agar

Ingredients	**Gm/litre**
Peptic digest of animal tissue	1.00
Dextrose	1.00
Monopotassium phosphate	0.80
Dipotassium phosphate	1.20
Sodium Chloride	5.00
Agar	15.00
Phenol red	0.012

Final pH (at 25^0C) 7.4 ± 0.2

Preparation (1000 mL): 24 gm of urea agar media was suspended in 950 mL of distilled water and dissolved completely by boiling. Then it was sterilized by autoclaving at 10 lbs pressure (115^0C) for 20 minutes. Then, the medium was cooled to 50^0C and 47.5mL of sterile 40% urea solution was added aseptically and mixed well. The medium was dispensed in sterile tubes and allowed to set in the slanting position.

APPENDIX VII

Principle and procedure for gram staining and biochemical properties

Gram staining:

Principle: -

Gram staining is a very useful differential staining technique for identifying and classifying bacteria into two major groups: Gram positive and Gram negative. In this process, the bacterial smear is subject to four different reagents in the order: crystal violet (primary stain), Gram's iodine solution (mordant), acetone alcohol (decolorizing agent) and safranine (counter stain). The bacteria which retain the primary stain (appear dark blue or violet) are called Gram positive, whereas those that lose the crystal violet and counter stained by safranin (appear red/pink) are referred as gram negative.

Procedure: -

1. A clean grease free slide was taken and a thin smear sample was made on the slide. Then the slide was air dried and heat fixed.
2. The smear was covered with crystal violet for 1 minute and then washed with water.
3. Then the smear was covered with Gram's iodine for 1 minute and washed.
4. Then decolorized with acetone alcohol (10-15 seconds) and then washed with water.
5. Finally, the slide was covered with safranine for 1 minute and washed with water.
6. Then the slide was air dried and observed under microscope at 10x, 40x and 100X (oil immersion).

Catalase test

Principle: -

This test is used to differentiate those bacteria that produce the enzyme catalase, such as Staphylococci, from non-catalase producing bacteria such as Streptococci.
Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old.

Procedure: -

1. Using wooden stick, a portion of colony was transferred to the surface of clean, dry glass slide.
2. A drop of 3% H_2O_2 was added over the transferred colony.
3. Then effervescence of the gas was marked by bubbles in case of positive result within 10 seconds

Oxidase test

Principle: -

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple colour end product.

Procedure: -

1. A piece of oxidase paper was taken on clean glass slide (filter paper soaked with the oxidase reagent 1% tetra methyl- p- pheny lenediamine dihydrochloride).
2. A small portion of bacterial culture was taken with the help of wooden applicator and rubbed on the reagent paper.
3. Then oxidase paper was observed for the development of purple color within 15 seconds.

Oxidative-fermentative test

Principle: -

The oxidative-fermentative test determines metabolism of glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in Hugh-leifson media from green to yellow in the presence or absence of oxygen. During aerobic repiration, organisms metabolize glucose producing weak acid during glycolysis and Krebs cycle which in turn change the bromothymol blue indicator in Hugh-leifson media from green to yellow in the presence of oxygen.

Procedure:

1. Two tubes containing Hugh-leifson media 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 37^0C for 24-48 hrs and observed for color change in both the tubes.

Sulfide Indole Motility (SIM) test

Principle: -

The SIM media is a multi-test agar used to test for indole production while simultaneously determining motility and hydrogen sulfide production. Bacteria containing tryptophanase enzyme hydrolyzes the tryptophan present in the media to indole, which on addition of Kovac's reagent produces a layer of cheery red colour. Bacteria containing cysteine desulfurase break down the sulphur contacting amino acid like cysteine producing hydrogen sulfide during putrification.

Procedure:-

1. The organism was stabbed into the SIM media with the help of sterile inoculating wire and incubated at 37^0C for 24 hrs.
2. After incubation, 4-8 drops of Kovac's reagent was added to the tube and let it stand for a while.
3. The tube was observed for the development of cherry red color on the surface, as well as blackening of the media and motility of the bacterial growth.

Methyl Red (MR) test

Principle:-

In methyl red test, the test bacteria are grown in a broth media (MR media) containing glucose. The bacteria with the ability of utilizing glucose producing stable acid, change the colour of methyl red media to red in addition of methyl red reagent.

Procedure:-

1. MR-VP broth was aseptically inoculated with the organism with the help of sterile inoculating loop and incubated at 37^0C for 24 hrs.
2. Then 5-6 drops of Methyl red reagent were added to the incubated test tubes.
3. The positive test is indicated by the development of red color.

Voges-Proskauer (VP) test

Principle:-

The VP test is used to determine if an organism produces acetylmethyl carbinol from glucose fermentation. If acetylmethyl carbinol is present, it is converted to diacetyl in the presence of alpha-napthol, strong alkali (40% KOH), and atmospheric oxygen. The diacetyl and quinidine containing compounds found in the peptones of the broth then condense to form a pinkish red polymer.

Procedure: -

1. The bacterial suspension was inoculated aseptically into the MR-VP broth tube and incubated at 37^0C for 24 hours.
2. Then, the Barrit's reagent A (alpha-napthol) and B (Potassium hydroxide) were added in the ratio of 3:1 and the tube was shaken.
3. The tube was observed for the development of red color after incubation at 37^0C for 20-30 minutes aerobically.

Citrate utilization test

Principle:-

Citrate utilization test is used to differentiate among enteric bacteria on the basis of their ability to ferment citrate as the sole carbon source. Simmon's citrate agar contains sodium citrate, the only source of carbon and energy for growing organism. Bromothymol blue is used as an indicator. When the citric acid is metabolized, the CO_2 generated combines with the sodium and water to form sodium carbonate an alkaline product, which changes the color of the indicator from green to blue and this constitutes a positive test. Bromothymol blue is green when acidic and blue when alkaline.

Procedure:-

1. The organism was streaked aseptically on the surface of the Simmon's citrate agar slant and incubated at 37^0C for 24-48 hours.
2. The tube was observed for the change in the color of the media and growth was observed.

Triple sugar iron (TSI) agar test

Principle:-

Triple Sugar Iron (TSI) agar media is composed of three sugars; lactose 1%, sucrose 1% and glucose 0.1%, iron (Ferrous sulfites) and phenol red as an indicator. The indicator is

employed for the detection of fermentation of sugar indicated by the change in color of the media due to the production of organic acid and Hydrogen sulphide (H_2S). If an organism ferments any of three sugars or any combination of them, the media will be yellow due to the production of acid as end product of fermentation. Glucose utilization occurs both aerobically on slant where O_2 is available and in the butt where the condition is anaerobic. Change of colour slant and butt indicates production of gas from the fermentation of sugar by an organism is indicated by the appearance of bubbles in the butt or pushing up of the entire slant from the bottom of the tube. Bacteria while non fermenter of lactose and sucrose, doesnot change the colour of media i.e media remain red. Colour of Hydrogen sulphide (H_2S) production by an organism is indicated by the reduction of ferrous sulfites of the media to ferric sulphide, which is manifested as a black precipitate.

Procedure:-

1. Test organism was inoculated in the TSI slant by stabbing in the butt first and streaking on the surface of the slant using sterile inoculating wire.
2. The TSI slant was then incubated at 37^0C for 18 hours.
3. Then the color of change in the butt and slant was observed along with gas production and H_2S production.

Urea hydrolysis test

Principle:-

Urea hydrolysis test is used to determine the ability of certain microorganism to produce the enzyme urease. Organism capable of producing enzyme urease catalyzes the breakdown of urea into ammonia and carbon dioxide which cause the rise in the pH of the media. As the pH increases, the color of the phenol red changes from an orange to deep pink.

Procedure:-

1. The test organism was streaked on the surface of the urease agar slant and incubated at 37^0C for 24-48 hours.
2. The change in color of the media was observed.

APPENDIX VIII

Procedure for antibiotic susceptibility testing

1. The isolated colony was transferred to 2-3mL nutrient broth and was incubated at 37^0C for 4 hours.
2. Thus, prepared inoculum was compared with the 0.5 McFarland standard tube which gives evenly spread semi confluent growth.
3. Then sterile cotton swab was dipped into the broth containing culture and was spread over dried MHA plates with the help of sterile forceps.
4. The antibiotics placed on the agar with the help of sterile forceps and pressed gently.
5. The plate was left for a few minutes at room temperature for diffusion of antibiotic from the disc, and then it was incubated at 37^0C for 18-24 hours.
6. The susceptibility pattern was noted following the incubation by measuring zone of inhibition as sensitive, intermediate and resistant as according to CLSI guideline 2018.

APPENDIX IX

Preparation of MacFarland

McFarland turbidity standards

McFarland standard No.	1.0% Barium chloride (mL)	1.0% Sulfuric acid (mL)	Approx. cell density (1×10^8 cfu/mL)
0.5	0.05	9.95	1.5
1	0.1	9.9	3.0
2	0.2	9.8	6.0

Preparation of turbidity standard equivalent to McFarland 0.5

1. 1% v/v solution of Sulphuric acid was prepared by adding 1mL of concentrated sulphuric acid to 99 mL water and mixing it well.
2. 1% w/v solution of barium chloride was prepared by dissolving 0.5gm of dihydrate barium chloride ($BaCl_2 . 2H_2O$) in 50 mL of distilled water.
3. Then, 0.6 mL of the barium chloride solution was added to 99.4 mL of the sulphuric acid solution and mixed.
4. A small volume of the turbid solution was then transferred to a screw capped bottle.
5. To standardize the inoculum density for a susceptibility test, a $BaCl_2$ turbidity standard, equivalent to a 0.5 McFarland standard was used.

APPENDIX X

Morphological and cultural characteristics of bacteria

1. *E. coli*

Escherichia coli is one of the predominant enteric species in the human gut and, as part of the normal intestinal flora. It is gram negative, rod of 1 -33µm×0.4-0.7µm size, aerobic and facultative anaerobic, non-spore forming, motile, non- capsulated. On MacConkey agar colonies are pink, circular, moist, smooth, entire, margin and flat. Lactose fermenter. On VRBA, colonies are purplish red, lactose fermenter.

2. *Klebsiella* spp.

Klebsiella spp. are ubiquitous in nature and commonly are in water and food. It is gram negative, short, thick rod of 1-2×0.5µm in size capsulated, non- sporing, non- motile. *Klebsiella* spp are lactose fermenter.

3. *Citrobacter freundii*

Citrobacter freundii is another opportunistic pathogen, but also is a resident of the human gastrointestinal tract. It is gram negative, rod shaped, non sporing.

Source: Chakraborty (2019); Cheesebrough (2006) and Forbes, *et al*., (2004).

APPENDIX XI

Total coliform count (TCC, at 37^{0}C) and Thermotolerant coliform count/ Fecal coliform count (FCC, at 44^{0}C)

Sample code	Location	TCC (cfu/gm)	FCC (cfu/gm)
P1	Naikap	4.6×10^4	2.6×10^4
P2		4×10^1	-
P3		1.4×10^3	1.4×10^2
P4		2.3×10^4	1.4×10^3
P5		4.5×10^5	2×10^3
P6		1.3×10^5	9.6×10^4
P7	Kalanki	7.4×10^2	2×10^1
P8		7×10^1	-
P9		5×10^1	-
P10		2.7×10^5	1.9×10^4
P11		1.4×10^3	1.2×10^3
P12		1.3×10^5	5.7×10^4
P13	Bafal	2.1×10^5	1.5×10^4
P14		1×10^2	-
P15		1.8×10^5	1.1×10^3
P16		5.6×10^2	1.4×10^1
P17		-	-
P18		1.2×10^3	1×10^1
P19	Sitapaila	1×10^2	1×10^1
P20		1×10^5	1×10^3
P21		-	-
P22		-	-
P23		-	-
P24		2.4×10^1	-
P25	Thamel	1.5×10^5	1×10^1
P26		1.7×10^5	1.2×10^4
P27		2	-
P28		1.8×10^4	-
P29		-	-
P30		2×10^2	-

APPENDIX XII

Quality of paneer in different locations for coliforms

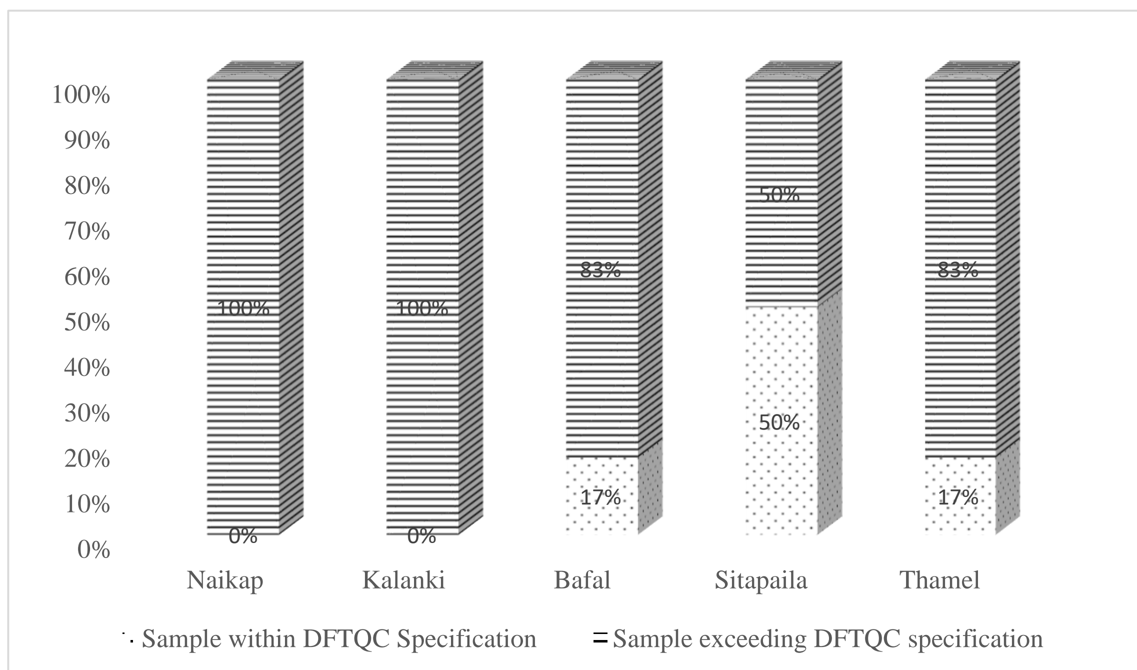

Figure 'A': Quality of paneer in different locations according to DFTQC specification of coliforms

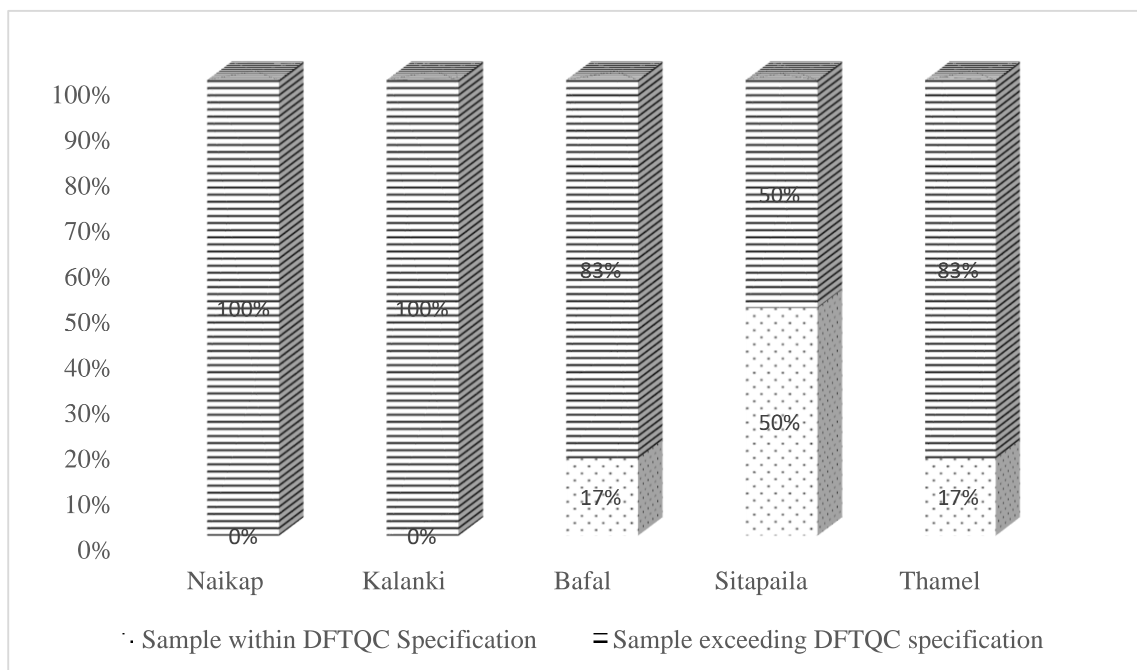

Figure 'B': Quality of paneer in different locations according to FSSAI specification of coliforms

APPENDIX XII

Biochemical tests for identification of bacteria

Test	Organisms			
	E. coli	***Klebsiella oxytoca***	***Citrobacter* spp.**	***Klebsiella* spp.**
Catalase test	+	+	+	+
Oxidase test	_	_	_	_
Oxidative fermentative test	F	F	F	F
Motility	+	_	+	_
Indole test	+	+	±	±
Methyl red	+	_	+	_
Voges Proskauer	_	+	_	+
Citrate utilization test	_	+	+	+
TSI test	Y/Y H2S- Gas+	Y/Y H2S- Gas+	Y/Y H2S-/+ Gas+	Y/Y H2S- Gas+
Urea hydrolysis test	_	+	±	+

+ = Positive, - = Negative, F= Fermentative, Y= Yellow

APPENDIX XIII

Bacteria isolates from different paneer samples

S.No	Location	Sample Code	Organism isolated at 37^0C
1.	Naikap	P1	*Citrobacter* spp.
		P2	*Klebsiella* spp.
		P3	*Klebsiella* spp.
		P4	*E.coli*
		P5	*E.coli*
		P6	*Citrobacter, Klebsiella* spp.
2.	Kalanki	P7	*E.coli,Klebsiella* spp.
		P8	*E.coli, Klebsiella* spp
		P9	*Citrobacter* spp.
		P10	*Klebsiella* spp.
		P11	*E.coli*
		P12	*E.coli*
3.	Bafal	P13	*E.coli*
		P14	-
		P15	*E.coli*
		P16	*E.coli*
		P17	*Citrobacter* spp., *E.coli, Klebsiella* spp.
		P18	*E.coli*
4.	Sitapaila	P19	*Klebsiella* spp.
		P20	-
		P21	*Citrobacter* spp., *E.coli, Klebsiella* spp.
		P22	*Klebsiella* spp.
		P23	*E.coli*
		P24	-
5.	Thamel	P25	*Klebsiella* spp.
		P26	*E.coli*
		P27	-
		P28	-
		P29	-
		P30	*E.coli, Klebsiella* spp.

Contd.

S.NO	Location	Sample Code	Organism isolated at 44^{0}C
1.	Naikap	P1	*Citrobacter* spp.
		P2	-
		P3	-
		P4	*E.coli*
		P5	*E.coli*
		P6	*Klebsiella* spp., *E.coli*
2.	Kalanki	P7	-
		P8	-
		P9	*Citrobacter* spp.
		P10	*Klebsiella* spp.
		P11	*E.coli*
		P12	*E.coli*
3.	Bafal	P13	*E.coli*
		P14	-
		P15	*E.coli*
		P16	-
		P17	-
		P18	*E.coli*
4.	Sitapaila	P19	*Klebsiella* spp.
		P20	*Klebsiella oxytoca*
		P21	-
		P22	*Klebsiella oxytoca*
		P23	*E.coli*
		P24	-
5.	Thamel	P25	-
		P26	*E.coli*
		P27	-
		P28	-
		P29	-
		P30	-

APPENDIX XIV

Zone size interpretative chart

For Enterobacteriaceae,

Antibiotic disc	Code	Disc content (mcg)	Zone of Inhibition (mm)			
			Sensitive	Intermediate	Resistant	*E. coli* ATCC 25922
Gentamycin	GEN	10	15	13-14	12	19-26
Cefoxitin	CX	30	18	15-17	14	23-29
Chloramphenicol	C	30	18	13-17	12	21-27
Tetracycline	TE	30	15	12-14	11	18-25
Nalidixic acid	NA	30	17	15-16	14	20-25
Cotrimoxazole	COT	25	16	11-15	10	23-29
Amikacin	AK	30	17	15-16	14	19-26
Ampicillin	A	10	17	14-16	13	15-22
Azithromycin	AZM	15	18	14-17	13	-
Levofloxacin	LE	5	17	14-16	13	29-37

Source: (CLSI 2013, CLSI 2018)