ANTIBIOTIC SUSCEPTIBILITY TESTING OF ISOLATED *Staphylococcus aureus* FROM PANEER SOLD 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 recommend that **Vagyashree Khanal** Symbol no. 500330043 T.U.Registration No.5-2-33-188-2017 has carried out project work entitled "**Antibiotic susceptibility testing of isolated *Staphylococcus aureus* from Paneer Sold in Kathmandu** "for the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology under my supervision in the Department of Microbiology, Amrit Campus Institute of Science and Technology (IoST), Tribhuvan University (T.U) Nepal.

To my knowledge, this work 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, Nepal for the submission of the project work for the partial fulfillment of Bachelor of Science (B.Sc.) degree.

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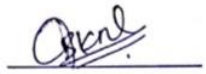
DECLARATION

The project work entitled "**Antibiotic susceptibility testing of isolated *Staphylococcus aureus* from Paneer sold 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 is carried out by me under the supervision of Mr. Atmaz Kumar Shrestha and co- supervision Asst. Prof. 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 the 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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LETTER OF FORWARD

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On the recommendation of **Mr. Atmaz Kumar Shrestha** and **Asst. Prof. Suchitra Thapa** this project work is submitted by **Vagyashree Khanal** Symbol No. 500330043 T.U. Registration No. 5-2-33-188-2017, entitled in**"Antibiotic Susceptibility Testing of isolated *Staphylococcus aureus* from Paneer Sold 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 *Staphylococcus aureus* from Paneer sold from Kathmandu District"** by Vagyashree Khanal, Symbol No. 500330043, T.U Registration No. 5-2-33-188-2017 under the supervision of Mr. Atmaz Kumar Shrestha and co-supervision Asst. Prof. Suchitra Thapa in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST),Tribhuvan University(T.U), is here by submitted for the partial fulfillment of the Bachelor of Science (B.Sc.) degree in Microbiology. This report has been accepted and forwarded to the Controller of Examination, Institute of Science and Technology, Tribhuvan University, Nepal for the legal procedure.

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ABSTRACT

Paneer is a fresh acid-set, non-aged, non-melting soft indigenous food made from curdling milk. Safety of dairy products is an issue of public health in Nepal. *Staphylococcus aureus* is an food borne pathogen of importance in dairy products. The main aim of this study is to detect microbial quality of paneer marketed in different localities of Kathmandu district. In present study, 30 paneer samples were randomly collected from dairy shops from 5 different locations in Kathmandu (Bafal, Kalanki, Naikap, Sitapaila and Thamel) during April to May 2022 and processed at laboratory of of Microbiology, Amrit Campus. Microbial analysis of paneer was done by Total bacteria count and total Staphylococcal count *S. aureus* were identified by biochemical tests and their antibiotic susceptibilty test was done by Kirby-Bauer disc diffusion method. Results obtained from this investigation revealed that out of 30 samples, 39.9% in Total Bacterial Count (TBC) was above specification. Among 30 samples, 29 samples showed *Staphylococcus* spp. growth and of those only 12 confirmed as *Staphylococcus aureus* . Among them 6 (20 %) exceeded specification of 100 cfu/gm. Antimicrobial susceptibility pattern of *S. aureus* showed 100% sensitivity towards Cotrimoxazole and Gentamycin and Levofloxacin, whereas resistance towards Chloramphenicol (8.3%), Cefoxitin (8.3%), Tetracycline(8.3%), Penicillin G (41.6%), Ampicillin (16.6%), Nalidixic acid (41.6%) and Azithromycin (16.6%). MRSA was detected in 1(3.34%). This study concluded that maximum number of sample exceeding microbial specification and antibiotic resistant isolates were reported from Naikap. Irresponsible and over use of antibiotics has lead to an increased the presence of multidrug Regular microbial quality monitoring of marketed paneer should be carried out by authorized Institution of Government of Nepal. Therefore, it is necessary to monitor and control the quality marketed in Kathmandu.

Key words: Ampicillin, Antimicrobial susceptibility test, Cefoxitin, Paneer, *Staphylococcus aureus*,Total bacterial count

शोधसार

पनिर एक नरम स्वदेशि खाद्य हो जुन दुधले बनाइन्छ । दुग्ध जन्य पदार्थको सुरक्षा नेपालको जनस्वास्थ्यको समस्या हो । हालको अध्ययनमा काठमाडौंका विभिन्न स्थानबाट (बाफल, कलंकी,नायकाप, सितापाइला, ठमेल)अप्रिल देखि जुन महिना २०२२ सम्म ३० वटा पनीरको नमूना संकलन गरि तिनिहरुको गुणस्तर अमृत साइन्स क्याम्पसको माइक्रोबायोलोजी विभागको प्रयोगशालामा परिक्षण गरिएको थियो । यस अध्ययनको मुख्य उद्देश्य काठमाडौं जिल्लाका पनीर बजारमा रहेका स्थानहरूमा माइक्रोबियल गुणस्तर पत्ता लगाउनु हो । *Staphylococcus aureus* डेयरी उत्पादनहरूमा महत्वको खाद्यजनित रोग जन्य ब्याक्टेरिया हो । पनीरको माइक्रोबियल विश्लेषण कुल गणना विधिद्वारा र कुल *Staphylococcus spp.*गणनविधिद्वारा गरिएको थियो । ग्राम स्टेनिङ र बायोकेमिकल परीक्षणद्वारा *Staphylococcus aureus* को कुल आइसोलेट्स पहिचान गरिएको थियो र किर्बी–बाउर डिस्क डिफुज्न विधिद्वारा तिनीहरूको एन्टिबायोटिक संवेदनशीलता परीक्षण गरिएको थियो। यस अनुसन्धानबाट प्राप्त नतिजाहरूले TBC मा ३९.९% निर्देशिका र मापढण्डभन्दा बढि भएको पाइएको छ । ३० नमुना मध्ये २९ नमुनाले *Staphylococcus spp.* वृद्धि देखाएको छ र ती मध्ये १२ वटा मात्रै *S.aureus* भएको पुष्टि भएको छ । ६ नमूनाहरू अर्थात २०% ले मानकमूलय १०० cfu/gm निर्देशिका र मापढण्डभन्दा बढि भएको पाइएको छ । *S. aureus*को एन्टिमाइक्रोबियल संवेदनशीलता Cotrimoxazole Gentamycin र Levofloxacin प्रति १००% देखायो जबकि Ampicillin (१६.६%), Azithromycin(१६.६%), Penicillin G (४१.६%),Cefoxitin (८.३%), Nalidixic acid (४१.६%), Tetracyclin (८.३%) र Choramphenicol (८.३%) प्रति प्रतिरोधी देखायो । पनीरबाट अलग गरिएको १ (३.३४%)*S.aureus* MRSA को रूपमा पत्ता लगाइयो । यो अध्ययनले नैकाप क्षेत्र ब्याक्टेरियाको अधिकतम संख्या र उच्च एन्टिबायोटिक प्रतिरोधी भएका नमूना पत्ता लागेको निष्कर्ष निकालेको छ । एन्टिबायोटिकको गैरजिम्मेवार र अत्याधिक प्रयोगले बहुऔषधि प्रतिरोधी जीवहरूको उपस्थिति बढेको छ । बजारमा पाइने पनीरको नियमित माइक्रोबियल गुणस्तर अनुगमन नेपाल सरकारको आधिकारिक संस्थाबाट गरिनुपर्छ ।

मुख्य शब्दहरू पनिर, *Staphylococcus aureus*, antimicrobial संवेदनशीलता परीक्षण, कुल ब्याक्टेरिया गणना, Cefoxitin, Ampicillin

LIST OF ACRONYMS AND ABBREVIATION

WHO	World Health Organization
cfu	Colony Forming Unit
CHBS	China Health and Birth Control Statistics
CLSI	Clinical and Laboratory Standard Institute
DFTQC	Department of Food Technology and Quality Control
SE	Staphylococcal Enterotoxins
FBD	Foodborne Disease
FDA	Food and Drug Administration
FSSAI	Food Safety Standardization and authority of India
gm	Gram
HACCP	Hazard Analysis and Critical Control Point
HA-MRSA	Hospital Acquired Methicillin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
NB	Nutrient Broth
MRSA	Methicillin Resistant *Staphylococcus aureus*
MA	MacConkey Agar
mcg	Microgram
MDR	Multi Drug Resistant
MHA	Muller Hinton Agar
Mm	Millimeter
Ml	Milliliter
ATCC	American Type Culture Collection

LIST OF SYMBOL

&	And
%	Percentage
×	Multiply
>	Greater than
^{0}C	Degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Milk is defined to be the lacteal secretion, practically free from colostrums, obtained by complete milking of one or more healthy cows which contains not less than 8.5% milk solids-not-fat and not less than 3.5 % milk fat (USPHS, 1965). Milk is a notorious as well as balanced food rich in proteins, fat, carbohydrates, vitamins, minerals and essential amino acids (Maharjan *et al.*,2014).Milk is used for production of different dairy products like yoghurt, ghee, butter, cheese, khuwa, paneer, chhana, ice cream and milk powder. Paneer represents a South Asian variety of soft cheese prepared by acid and heat coagulation of milk (Khan, &Pal 2011). It contains all the milk constituents except loss of some soluble whey proteins, lactose and minerals. 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).It is of great value in diet, especially in the South Asian countries including Nepal and Indian vegetarian context, because it contains a fairly high level of fat and proteins as well as some minerals, especially calcium and phosphorus. It is an excellent source substitute for meat in diet in vegetarian cuisine (Khan and Pal 2011).

Milk contamination can occur in many different ways. Some ways include: Cow feces coming into direct contact with the milk. Infection of the cow's udder (mastitis) Cow diseases (e.g., bovine tuberculosis) and bacteria that live on the skin of cows. Environment (e.g., feces, dirt, processing equipment). Milk secreted from uninfected animals udder is almost sterile and invariably, it becomes contaminated during milking, cooling or storage. It is an excellent medium for the growth of bacteria, yeasts and molds that are the common contaminants of any food materials. Their rapid growth, particularly at high ambient temperatures can spoil the milk for liquid consumption and for manufacturing of dairy products (Poutrel *et al.*,2015). The main zoonotic pathogens identified in raw milk were *Brucellaspp*., mainly *Brucella melitensis*, *Listeria monocytogenes*, *Salmonellaspp*., *Mycobacteriu bovis*, *Yersinia enterocolitica*, *Streptococcus pyogenes* and *Streptococcus agalactiae*, and *Escherichia coli* O157:H7 and *Enterobacter sakazakii*.

Most foodborne diseases are infections caused by a variety of bacteria, viruses, and parasites (Havelaar and Grace 2020).Many bacterial pathogens such as *Escherichia coli, Salmonella* Typhimurium, and *H. pylori* can circumvent the acid conditions of the stomach by developing adaptive mechanism that allow these bacteria to survive in acid environment (Smith, 2003).Staphylococcal food poisoning is characterized by sudden start of nausea, vomiting and stomach cramps, diarrhea. 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).Food contamination with antibiotic-resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance. Bacteria can become resistant by genetically to resist the effect of an antibiotic. They also can be resistant after getting a resistance gene from antibiotic resistant bacteria (Redfield, 2019).

Careful food preparation can prevent staphylococcal food poisoning. Treatment of staphylococcal food poisoning usually consist of drinking an adequate amount of fluids (Gotfried, 2021)

1.2 Rational of Study

This study is crucial to identify the hazardous microorganisms present in paneer and their antibiotic susceptibility testing. Dairy consumers need to be informed about various contaminated products, foodborne microorganisms, and ailments brought on by contaminated food. Therefore, the study contributes to consumer awareness of the danger of foodborne illness. Food poisoning and diarrhea can result from consuming paneer that is of poor microbial quality. In the dairy industry, routine monitoring is crucial. The purpose of this study to locate the market where poor quality paneer is sold and to encourage consumers to purchase safe paneer products.

1.3 Objectives

1.3.1 General objective

- To determine antibiotic susceptibility pattern of isolated *Staphylococcus aureus* from paneer marketed in different localities of Kathmandu.

1.3.2 Specific objectives

- To enumerate total viable bacteria from paneer sample
- To enumerate *Staphylococcus spp.* from paneer sample
- To isolate and identify total *S. aureus* from paneer sample
- To determine antibiotic susceptibility pattern of *Staphylococcus aureus* from paneer sample

CHAPTER 2

LITREATURE REVIEW

Paneer is the milk solids obtained by the acid coagulation of hot milk and subsequently age of whey the acid commonly used are citric ascetic and sour whey or cultured whey can also be used for coagulation of milk(Khan, *et al.*,2011).

2.1 History of paneer

Paneer also known as ponir or Indian cottage cheese, is a fresh acid set cheese common in the Indian subcontinent, made from cow and buffalo milk. It is non aged non melting soft cheese made by curdling milk with fruit or vegetable derived such as lemon juice (Kumar *et.al*., 2011). People have been consuming solid mass made from milk since kusana and Saka Satavahana periods (AD 75-300),hence this description of paneer as a solid mass seems to be earlier reference to the present day paneer (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).White paneer is a staple food of Nomads in Afghanistan(Srivastava and Goyal, 2007).

Cheese are manufactured by using high heat along with acid preparation without restoring to use of starter culture (similar to Indian paneer) was practiced in many countries of South Asia, Central South and Latin America.Most frequent potential bacterial pathogens associated with milk or dairy product in industrialized countries are *Staphylococcus aureus, Salmonella spp, Listera monocytogenes* and *Escherichia coli* O157;H7(Jackobsen, *et al.*,2011).

Singh and Singh, (2000) analyzed the market sample of paneer collected from Arga city and found comparatively lower total plate count (6.51 $\log_{10}$ cfu/ml), coliform count (3.05$\log_{10}$ cfu/ml) for laboratory made sample against 18.00,10.39 $\log_{10}$ cfu/ml respectively.

2.2 Paneer manufacturing process

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

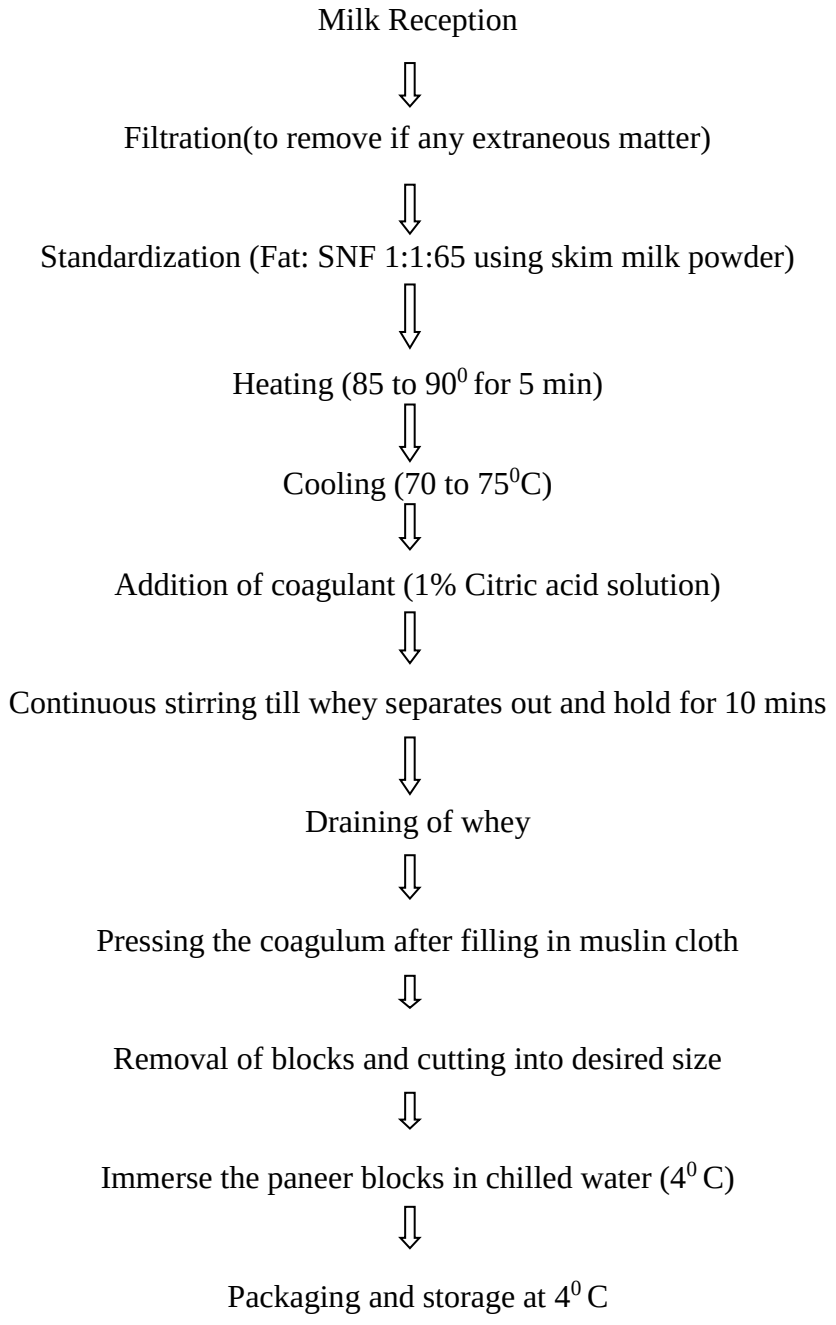


Fig:1 Flowchart of manufacturing of paneer (NIFTEM 2020)

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). 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 (Poudel *et al*, 2017). Preparation and selling of milk products are very less in a country.

Milk producing farmers are suffering from the problem of raw milk and pasteurized milk is very low as compared to different milk products. So to overcome the problem of milk holiday it will be the great idea to diversify milk into different products which will ultimately increase 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 selling prize of raw milk is similar(NCRP, 2019).Paneer packaged in high film (EVA/ EVA/ PVDC/ EVA) under vacuum and heat treated at 90^0C for one minute is reported to have a shelf life of 90 days under refrigeration Vacuum packaging is essentially a 'one size fits all' technology – it relies solely removing air (Singh, *et al*., 2019).

2.3 Bacteria found in dairy product

Milk and dairy products are important source of vital nutrients for human beings. The unique composition and properties make milk an excellent medium for bacterial growth and source of bacterial infection. Milk-borne pathogenic bacteria pose a serious threat to human health, and constitute about 90% of all dairy- related diseases. An outbreak survey of human gastrointestinal disease could be an epidemiological indicator of food-borne disease, which may be originated from drinking unpasteurized milk; *Salmonella* species can be found in ice cream and fresh cheeses as well as *Brucella melitensis* in non-pasteurized milk and homemade dairy products mostly goat cheese. The main zoonotic pathogens identified in raw milk were *Brucella*spp., mainly *Brucella melitensis*, *Listeria monocytogenes*, *Salmonella spp*., *Mycobacteriu bovis*, *Yersinia enterocolitica*, *Streptococcus pyogenes* and *Streptococcus agalactiae*, and *Escherichia coli* O157:H7 and *Enterobacter sakazakii* are recently reported. New emerging pathogens causing milk food-borne diseases are considered:,*Mycobacterium avium* subspp. *paratuberculosis*, *Streptococcus zooepidemicus* (β-hemolytic Streptococcus

Lancefield C group), *Campylobacter jejuni*, *Citrobacter freundii*, *Corynebacterium ulcerans* (Mozsik and Figler, 2019).

2.4 Morphology and virulence factors of *Staphylococcus aureus*

Staphylococcal species are Gram positive non motile, catalase positive, small, spherical bacteria (cocci), which on microscopic examination, appear in pairs short chains,or bunched in grape like clusters. Staphylococci are ubiquitous and impossible to eradicate from the environment.

Staphylococcus aureus is both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus*. Simultaneously, it is a leading cause of bacteremia and infective endocarditis (IE) as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections. *S.aureus* produce many virulence factor such as capsular polysaccharide, cell wall associated with polymers, leukocidin, hemolycin extracellular enzyme and protein receptor (Oogai *et, al.*,2011, Chakraborty, 2016).

Several staphylococcal species, including both coagulase –negative and coagulase positive strains, have the ability to produce highly heat stable enterotoxins that cause gastroenteritis in humans. *S.aureus* is the etiologic agent predominantly associated with staphylococcal food poisoning. *S.aureus* is versatile human pathogen capable of causing staphylococcal food poisoning, toxic shock syndrome, pneumonia, postoperative wound infection and nosocomial bacteremia. *S.aureus* produce a variety of extracellular products, many of which act as virulence factors. Staphylococci are mesophilic. *S. aureus* growth in general ranges from 7^0C to 47.6^0C with 35^0 C being the optimum temperature for growth (FDA, 2012).

Growth of microbial pathogens in cheese is dictated by extrinsic and intrinsic parameters. The important intrinsic parameters include moisture content; pH and acidity; nutrient content, redox potential, presence of antimicrobial compounds, either those occurring naturally or those that are added as food preservatives(ICMSF, 1986). Another important factor affecting the growth of microorganism in the availability of the water for the growth and metabolic processes, termed as the water activity value (Valik *et, al.*,2009).A characteristic feature which distinguishes *S. aureus* from other pathogenic bacteria is its high tolerance to low water activity (a_w) values and NaCL

concentration of up to 20% . Generally, minimal a_w required fo *S. aureus* growth is 0.83-0.86 (Madvedova and Valik, 2012).

2.5 Foodborne disease

Foodborne disease are caused by contamination of food and occur at any stage of the food production, delivery and consumption chain. Foodborne diseases encompass a wide range of illness from diahorrea to cancer.Most present as gastrointestinal issues, though they can also produce neurological,gynological and immunological system(WHO 2022). Bacterial foodborne diseases (FBD) are the main reason for 2/3 of food poisoning outbreaks the majority of the countries. FBD is a matter of concern especially in developing countries like Nepal (Gautam *et al.* 2013).Bacteria that are responsible for foodborne disease are *Staphylococcus aureus*, *Salmonella spp,* toxigenic *Escherichia coli*, *Camphylobacter* (Havelaar *et al* 2015 and Ekici, *et al* 2018).

Food unsafe for consumption causes 600 million cases of food borne disease and 420000 death every year, and 56 million people die each year (Ritchie and Roser 2018, WHO, 2015). These data indicates that 7.69% (600 million) individuals of world populations (7.8 billion) suffer from foodborne disease every year and 7.5% (420000 death) of all death (56 million) annually are due to foodborne illness (WHO 2015).

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).

2.6 Epidemiology of foodborne disease outbreaks due to dairy product

Dairy production is rapidly increasing in developing countries and making significant contributions to health, nutrition, environments, and livelihoods, with the potential for still greater contributions. However, dairy products can also contribute to human disease in many ways, with dairy borne disease likely being the most important health risk may

biological, chemical, physical or allergenic hazards presents in milk and other products. (Grace and Havelaar, 2020).

Milk and dairy products represent excellent growth media for many spoilage and pathogenic microorganisms because of unique composition and properties (Nada, *et al.,* 2012 and Clayes *et al.*,2013). Unpasteurized milk can be a vehicle for verity of

microorganism (*Staphylococcus aureus, Listeria spp, Salmonella spp*. and *Camphylobactor spp*.) and that outbreaks is related to cheese made with unpasteurized milk (Binanchi,*et al*. 2013).

Ayers *et, al.,*2006 reported 121 outbreaks in United states,73(60%) involved non pasteurized products and resulted in 1571 cases,202 hospitalizations and 2 deaths. A total of 55 (75%) outbreaks occurred in 21 states that permitted sale of non pasteurized products, incidence of non pasteurized product associated outbreaks was higher in these states.

During 1998-2011, 90 outbreaks attributed to milk product were reported in US; 38 (42%) were due to milk products made with unpasteurized milk, 44(49%) made with pasteurized milk and the pasteurization status was not reported for the other eight(8%) (Gould, *et al.,*2014).

Nicholas *et al*. (2002) reported from 1973 through 1997, states and local health departments reported 604 outbreaks of foodborne disease in schools of united states.

2.7 Epidemiology of Staphylococcal food poisoning

Staphylococcal food poisoning is one of the most frequent foodborne illnesses worldwide and it is caused by the ingestion of food contaminated with enterotoxins produced by some strains of *Staphylococcus aureus* (Tondo,*et al*., 2013). Most of the cases occur in summer when temperature is and food is stored improperly (Montvile and Matthews 2005). The toxins of *S aureus* are known as enterotoxin because they are able to promote water loss from small intestinal mucosa and resulting in vomiting and diarrhea (Martin,*et al.,* 2003) Foodborne enterotoxin caused by consumption of foods contaminated with SEs considered of second most common foodborne illness in the world (Pinto,*et. al*. 2004).

The true incidence of *Staphylococcus aureus* food-borne disease (SFD) could be a lot higher as sporadic food-borne disease caused by *S. aureus* is not reportable in the United States.Some other contributing factors for the low incidence of SFD include misdiagnosis, improper sample collection and laboratory examination , lack of seeking medical attention by the affected persons complicating the laboratory confirmation , and lack of routine surveillance of clinical stool specimens for *S. aureus* or its enterotoxins (Kadariya,*et. al*.,2013).

Staphylococcal food poisoning (SFP) is a common cause of foodborne illness worldwide. SFP occurs following ingestion of staphylococcal enterotoxins which are heat resistant and are produced in food following contamination by staphylococci, typically *Staphylococcus aureus*. Foods including sliced meat, meat products, salads, pastries, custards, raw milk and cheese products present a particular contamination risk. Such a large population of staphylococci is indicative of unhygienic food handling procedures and temperature abuse over a period of time to allow for bacterial growth (Bates,*et. al.,* 2013).

In the United States (US) the notification rate for vancomycin-intermediate *S.aureus* was 0.04 cases per 100,000 populations in 2010, which was as increase from the 2009 rate of 0.03 NCHS (2013). It is estimated that in the US, *S. aureus* accounts for 2.6% of foodborne illness caused by 31 major pathogens (Scallan, *et al.*,2011).

2.8 *Staphylococcus aureus* in Paneer

Thaker, *et. al.*, (2013) performed a study on the isolation and identification of *S.aureus* from milk and milk products and their drug resistant pattern in Anand Gujrat and result revealed that out of 160 sample; milk (100),milk product(30), pedha 30 resulted in the isolation of 10 (6.25%) of *S. aureus.*

Tripathi, *et al.* (2018) studied different milk product sample. In paneer highest presence of *S.aureus* was in local retailers 30.0% followed by branded I and brand II with 13.3% each.

Singh and Singh (2000) were studied the sample of marketed paneer collected from Arga city and found comparatively lower plate count (6.52gm log cfu/gm).Egyptian soft cheese found that total aerobic bacterial count was $1.4\times10^5\pm 1.7\times10^5$ cells/gm and 47.5% tested cheese are free from coliform bacteria and *E.coli* 98.5%of the tested cheese free from *S.aureus* (Ghada,*et. al.*, 2004).

Marami,*et al*., (2022) reported that the prevalence of *S. aureus* in curd milk was higher in this study (24.14%), which is consistent with the 25.4% reported from the Tigray region, Ethiopia, and 21.1% in north-central and north-eastern Greece . However, the current study contradicts the findings of South-West Uganda (12.1%) ,Iran (0.00%) ,and Annand, Gujarat (3.33%) .

2.9 Antibiotic and antibiotic resistance

The increasing rates of drug resistance among pathogenic Gram-positive and Gram-negative bacteria have been well documented (Rolston and Bodey 2003). This resistance coupled with the rise in opportunistic infections and side effect of constant use of several synthetic antimicrobial agents have required a search for new and novel drugs of natural origin. Methicillin resistant *Staphylococcus aureus* (MRSA) is defined as any strains of *S. aureus* that has acquired resistance to Methicillin and other β-lactam antibiotics (Stotts, *et al*., 2005). Strains of Methicillin resistant *S. aureus* is conferred by the presence of the *mec*A gene (or its homologue *mec*C gene) encoding for the production of an altered Penicillin binding protein (PBP2a) with a low affinity for all β-lactam antimicrobials (Visciano, *et al*., 2014). The first Methicillin resistant *S. aureus* (MRSA) infection was described and since then, human infections caused by multi-drug resistant MRSA have become common (Waness, 2010).

2.10 Antimicrobial Resistance (AMR) in *S.aureus* in dairy products

Methicillin-resistant staphylococci (MRS) are resistant to all penicillin, including semisynthetic penicillinase-resistant congeners, penems, carbapenems, and cephalosporins. The basis of this resistance is conferred by an additional penicillin-binding protein (Roy, *et al.*, 2000).

Gautam, *et al.* (2015) studied 153 isolates of bacteria from dairy products and antibiotic susceptibility pattern of *S. aureus, E.coli* was determined. *E. coli*was sensitive towards Genthamycin, Ciprofloxacin and Ofloxacin intermediate against Tetracycline and Kanamycin and resistance towards Amoxicillin. Similarly, *S aureus* was sensitive for Genthamycin, Tetracycline, and Amoxicillin with intermediate against Ciprofloxacin.

Sasidgaran, *et al.*(2011) studied that 50 sample of dairy products were contaminated with *S aureus* and 5 isolateswere subjected to antimicrobial resistance pattern using five antibiotics discs (Methicillin, Vancomycin, Kanamycin, Choramphenicol and Tetracycline). Sample 29 showed resistance to Methicillin and Vancomycin.

Mashouf *et al.*(2015) studied 1050 food and milk products samples from Iran, 98 *S.aureus* strains were isolated. Out of the 98 isolates examined, the most frequent resistance was observed to Erythromycin, followed by Tetracycline, Gentamycin, Clindamycin, Ciprofloxacin and Rifampin, Sulfamethoxazole and Cefoxitin.

Rahimi (2013) studied 347 dairy products sample in Iran, out of which 20 samples were found to be contaminated with *S.aureus*. Isolates were resistant towards Tetracycline and Penicillin G.

Shrestha *et al*. (2021) studied 460 milk samples were screened using the California Mastitis Test (CMT) in Chitwan and positive samples were subjected to microbiological analysis to isolate and identify *S.aureus*. Out of 191 CMT positive milk samples, the biochemical test showed that the prevalence of *S. aureus* was 15.2%. Among the 29 *S.aureus* isolates 6.9% were identified MRSA.

NCRP 2019 the isolated *Staphylococcus* mostly resistance to Ampicillin, Amoxyclav and sensitive to Tetracycline followed by Chloramphenicol, Gentamycin, Cetriaxone and Ceprofloxacin. Similarly, Dhakal 2007 also found that mastitis pathogens have developed resistance to Ampicillin and Penicillin.

Timalsina *et al.*(2019) in study of 109 of dairy products sample *S.aureus* were isolated. Out of them 32 MRSA were isolated from dairy samples. Among of the MRSA isolates were susceptible to Gentamicin followed by Ciprofloxacin.

Rimal, *et al.*(2020) studied a total 70 milk samples from Kathmandu district, 30 isolates were identified as *S.aureus*. All *S.aureus* were susceptible to Choramphenicol were resistant to Cefoxitin, the presence of Methicillin resistant *S.aureus* (MRSA).

Karki, *et al.*(2014) studied 1173 clinical specimen from which 100 *S.aureus* were isolated of which 19% cases were MRSA. *S. aureus* showedresitance towards Vancomycin 100%, Amikacin (90%), Gentamycin (83%)and Tetracycline (81%).

2.11 Methods of quality evaluation of paneer

The quality paneer as a whole can be evaluated from different parameters as mentioned by Singh and Chandra (2017) includes chemical analysis sensory analysis and microbial analysis.

Sensory analysis evaluates the colour, texture and flavor(Srivastava and Goyal 2007). Good quality of paneer is characterized by marble white color,sweetish,mildly acidic taste nutty flavor spongy body and smooth texture (Khan and Pal 2011).

Microbial analysis includes yeast mold count and coliform count(Dongare *et al.*2019). In bacterial count, count of pathogenic bacteria like *Staphylococcus spp., Escherichia coli, Klebsiella spp., Listeria monocytogens, Salmonella spp.*(Girdharwal 2018).

Microbial Analysis

Aerobic Plate count, Yeast& Mold count, and Coliform count 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 are as follows. Given here are acceptable limits of total plate count (TPC), coliform, yeast and mould, *E. coli* and *S. aureus* for paneer.

Microbiological data for paneer

Microbes	**Minimum**	**Maximum**
Aerobic Plate Count	1.5 X 10^5/gm	3.5 X 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: Food Standard, FSSAI (2012)

The FSSAI regulations specify a permissible standard limit of microorganisms for Panner 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 DFTQC 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 colours and other substances and should have the following quality parameter(DFTQC 2010).

Quality Parameter of Panner

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

Source: Food Standard, DFTQC (2010)

According to Nepal standard, there should be absence of coliform in dairy products (coliform count should be zero) (DFTQC 2010)

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

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

3.2 Study area

Kathmandu is the nation's capital with the population of 20,17,532. Kathmandu District lies in Province 3 of Nepal. In this study, samples were collected from the four different locations i.e Bafal, Kalanki, Naikap, Sitapaila and Thamel of Kathmandu.

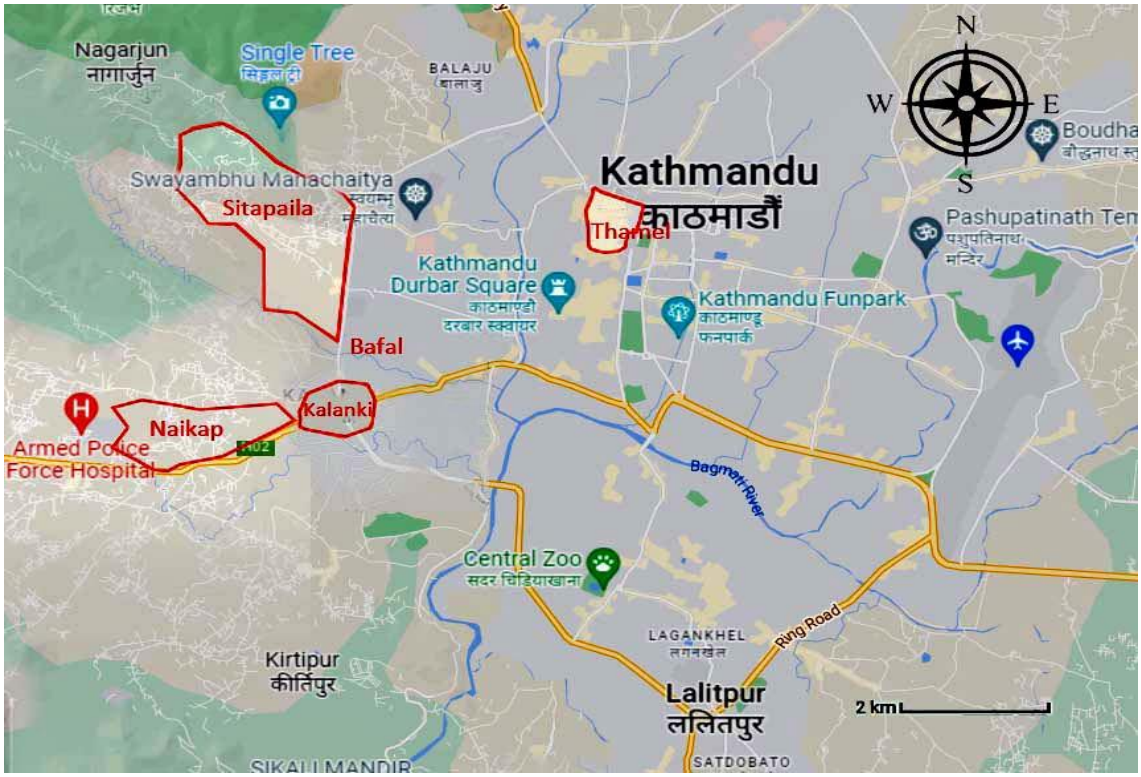


Fig 2: Geographical Map of Bafal, Kalanki, Naikap, Sitapaila and Thamel in Kathmandu

3.3 Methodology and study design

3.3.1 Type of study

The present study employed a Cross sectional experiment design to detect the microbial contamination in paneer.

3.3.2 Sample size and site

A 30 paneer sample was collected from different retail shop of Kathmandu district,sample was collected from different localities of Kathmandu. Convenient sampling was followed for sample collection.

3.3.3 Sample collection and Transportation

A total of 30 samples of paneer was collected from different localities of Kathmandu in pre-sterile foils maintaining 4^0C in icebox. The samples were transported in the laboratory within 2 hours of collection and processed within 30 minutes of collection.

3.4 Time framework

This project was complete in 3 months from April to June 2022.

3.5 Laboratory analysis and processing

All the laboratory analysis of paneer samples were done in microbiology laboratory of of Microbiology, Amrit Campus, Tribhuvan University. Detection of bacteria of paneer samples was determined by standard guideline of Food Safety Standards Authority of India (FSSAI). Total plate count (TPC) Total Staphylococcus count (TSC) and the isolation of *Staphylococcus spp.*. Determination of antimicrobial susceptibility of the isolated *Staphylococcus spp* were done.

3.5.1 Sample preparation and dilution

10gm of paneer sample was homogenized aseptically in 90ml sterile normal saline in a conical flask using vortex after that dilution upto 10^6 were done. Sample preparation method is in Appendix(II) Samples were examined according to APHA (2004); BAM (2016); ISO (1978); Haddad and Yamani, (2017) with slight modification.

3.5.2 Total bacterial count

For total bacterial count, an appropriate weight of paneer sample is homogenized with diluents in normal saline. The diluted samples were plated using pour plate technique on Plate count Agar (PCA) and incubated at 37^0C for 24-48 hrs for isolation and enumeration of total bacteria. Total bacteria were counted and calculated using formula. Plate count agar is equivalent to the medium recommended by APHA for the plate count of microorganism in dairy products and may also be used to determine sanitary quality of foods, water and other materials(APHA, 2004).

3.5.4 Total Staphylococcal count

For staphylococcal count,10 gm weight of paneer sample is homogenized with diluents, normal saline(Girdharwal, 2018). The diluted samples are plated using Spread plate technique on Manitol salt agar (MSA) and incubated at 37^0Cfor 24-48 hrs for isolation and enumeration of *S. aureus* and total staphylococcal were count and calculated using formula. Further study of colony morphology and Gram staining and biochemical tests are performed for identification of *Staphylococcal spp* and *S. aureus* (BAM, 2012).Procedure for gram staining and biochemical test is in Appendix(VI).

3.5.5 Isolation and identification of *Staphylococcus aureus*

According to BAM (2016), Hadded and Yamani (2017), Girdharwal (2018), isolation and identification of *Staphylococcus aureus* was done. An isolated golden yellow colony on MSA was subculture on MSA plate and incubated at 37^0C for 24 hours. Colony morphology, gram staining and various biochemical test were performed from this isolated culture according to (Cheesbrough 2016 and Forbes, *et al*., 2007) Biochemical test was done for identification of *S. aureus* were catalase test, oxidase test,oxidative fermentative test and coagulase test.

3.5.6 Antibiotic susceptibility testing

Antibiotic susceptibility testing of isolated *S.aureus* was done following the Kirby – Bauer disk diffusion method on Muller Hinton Agar (MHA) (CLSI, 2018). The antimicrobial agents tested against *Staphylococcus spp* were Levofoxacin (5mcg), Tetracycline(30mcg),Cotrimoxazole(25mcg),Cefoxitin(30mcg),Ampicillin(10mcg),Chloramphenicol(30mcg), Ampicillin(10mcg), PenicillinG(10mcg), Gentamycine(10mcg), Nalidixic acid(30mcg) Plate was incubated at 37^0C for 24 hours. After incubation, the

zone of inhibition diameter was measured in millimeter scale. The zone of diameter for individual antimicrobial agents was then translated into sensitive, intermediate and resistant categories according to recommended interpretation guideline for common pathogens (CLSI, 2018).

3.6 Quality Control

All the necessary quality controls of media and culture plates were performed during the experiment. The media lot number and expiry date were also checked. *Staphylococcus aureus* (ATCC 43300) was taken as control.

For the best interpretation of results and recognition of any source of error in disc diffusion sensitivity methods the correct use of control was taken. Thus control strains *Staphylococcus aureus* (ATCC 43300) and *Escherichia coli* (ATCC 25922) were used for the standardization of the Kirby Bauer test by correct interpretation of the zone diameters.

3.7 Data analysis and Statistical Tools

All the results obtained in this study were entered in to the worksheet of MS EXCEL 2007 software and laboratory data was analyzed.

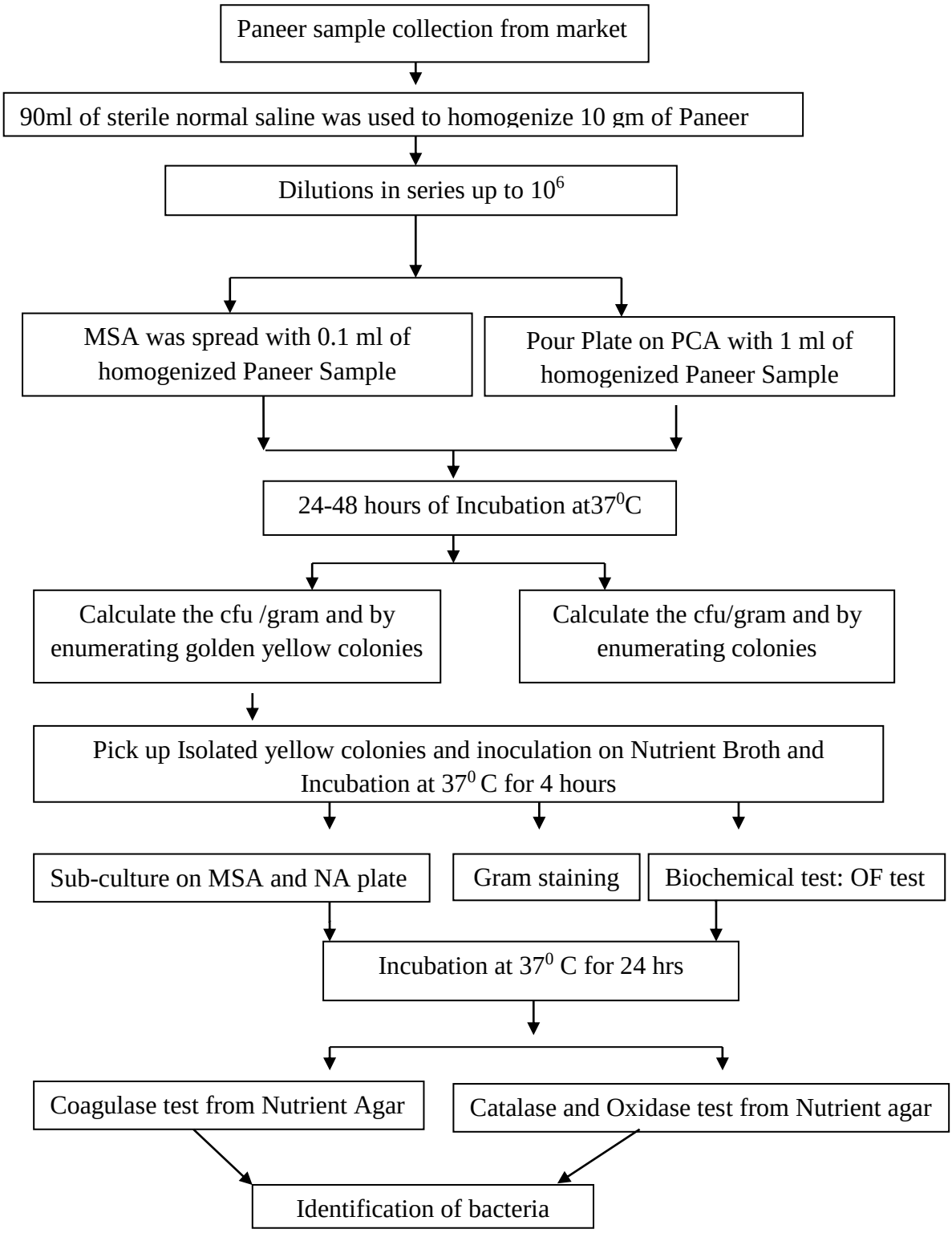


Fig 3: Flowchart of total bacterial count and isolation and identification of *Staphylococcus aureus* (BAM 2016, Cheesbrough 2016, Forbes, *et al*., 2007 Girdharwal 2018, Haddad and Yamani 2017,ISO 1978, Wehr and Frank, 2004.)

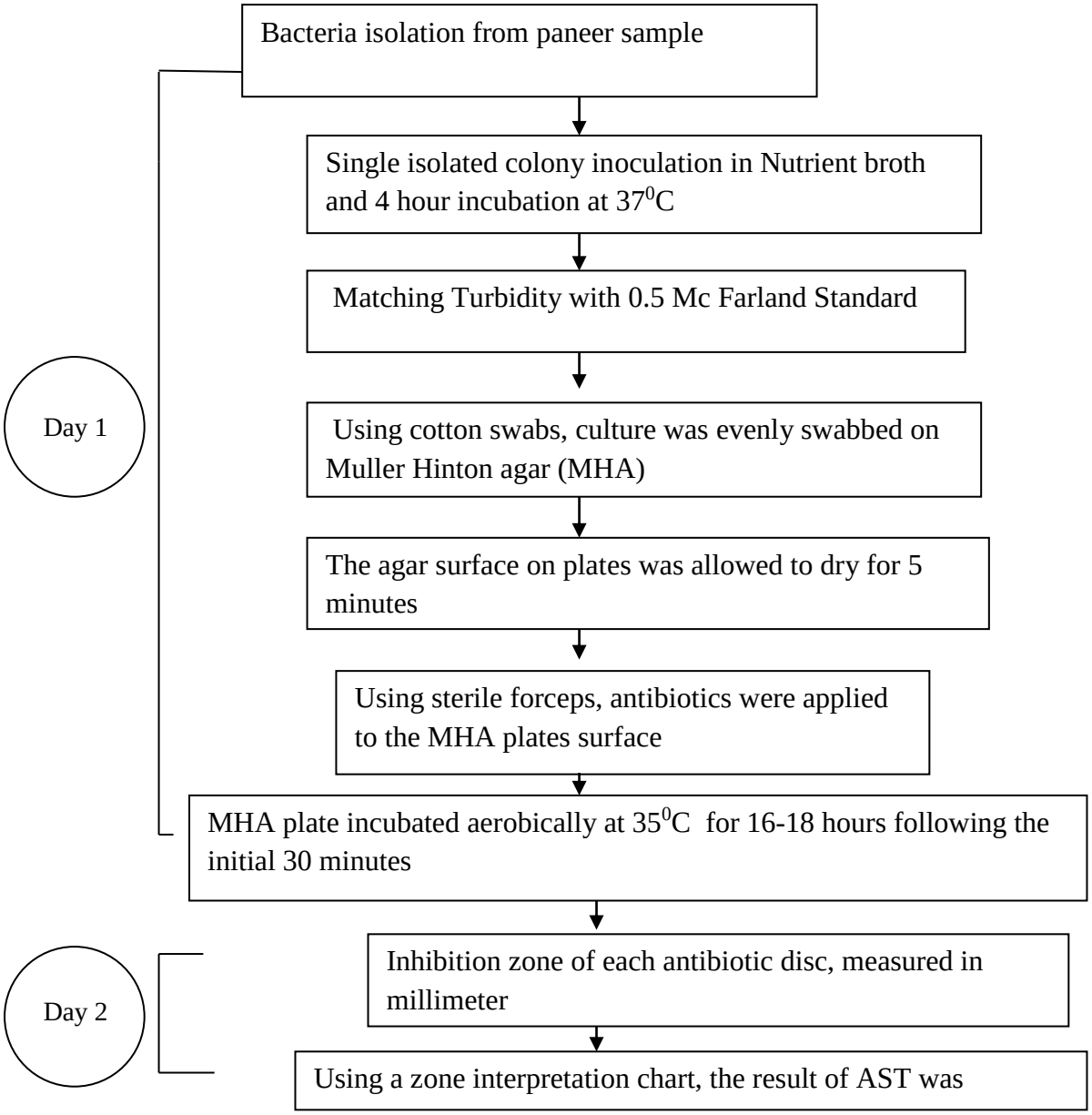


Fig 4: Flowchart for antibiotic susceptibility testing of *S.aureus i*solated from paneer (CLSI 2018)

CHAPTER 4

RESULT AND DISCUSSION

4.1 Result

A total of 30 paneer samples from five different localities of Kathmandu district (Naikap, Kalanki, Bafal, Sitapaila, Thamel) assessed for detection of total bacteria, *S.aureus* and their antibiotic susceptibility pattern was determined.

4.1.1 Total bacterial count

Total bacterial count on PCA was from paneer sample from different localities of Kathmandu represented on Table 1. The highest maximum count 9×10^6cfu/gm was found in paneer from Naikap .

Table 1: Location wise distribution of total bacterial count

Location	No. of sample	Minimum count	Maximum count	Average cfu/gm
Bafal	6	6.8×10^2	3×10^6	8.8×10^5
Kalanki	6	1.3×10^2	5×10^5	2.5×10^5
Naikap	6	1.3×10^3	9×10^6	3.1×10^6
Sitapaila	6	2×10^1	4×10^5	2×10^5
Thamel	6	1.9×10^2	8×10^6	6.6×10^5
Total	30			

Out of 30 samples, 12 samples had a total bacterial count that was above the acceptable limit given by FSSAI. Distribution of quality of paneer sample according to locations represented on Table 2. The percentage of sample within the maximum acceptable limit was in Sitapila (16.7%) followed by Kalanki (13.4%), Bafal (13.4%), Thamel (10%) and Naikap (6.6%) respectively. The TBC exceeded the maximum acceptable level (3.5×10^5 cfu/gm) in 39.9% of the samples.

Table 2: Location wise distribution of quality of paneer

Location	Bacteria presence sample	Sample within acceptable limit <1.5x10^4 cfu/gm		Sample exceeding acceptable limit >3.5x10^5 cfu/gm	
		No.	%	No	%
Bafal	6	4	13.4	2	6.6
Kalanki	5	4	13.4	2	6.6
Naikap	6	2	6.6	4	13.4
Sitapaila	6	5	16.7	1	3.3
Thamel	6	3	10	3	10
Total	30	18	60.1	12	39.9

4.1.2 Total count of *Staphylococcus spp* from paneer sample

Total count of *Staphylococcus spp* from paneer sample from different localities were represented on Table 3. The maximum count 8×10^5 cfu/gm was found in paneer sample from Naikap.

Table 3: Location wise count of total *Staphylococcus spp*

Location	No.of Sample	Minimum count	Maximum count	Average cfu/gm
Bafal	6	2×10^1	5.6×10^5	2×10^5
Kalanki	6	1×10^1	2.4×10^5	1.8×10^5
Naikap	6	2×10^1	8×10^5	4×10^5
Sitapaila	6	3×10^1	4×10^3	2.9×10^2
Thamel	6	1.2×10^1	2.6×10^4	1.3×10^4
Total	30			

4.1.3 Number of samples with *Staphylococcus aureus*

Out of 30, 29 samples showed growth for *Staphylococcus spp*. *Staphylococcus aureus* was isolated in 12 samples and other staphylococci was isolated in 17 samples. Total number of *Staphylococcus aureus* and other staphylococci was represented on Table 4.

Table 4: Location wise count of total number of *S.aureus* and other staphylococci

Location	*Staphylococcus aureus* sample		Other staphylococci	
	n	%	n	%
Bafal	2	6.67	4	13.34
Kalanki	2	6.67	3	10
Naikap	4	13.4	2	6.7
Sitapaila	2	6.67	4	13.34
Thamel	2	6.67	4	13.34
Total	12	40.2	17	56.9

4.1.4 Total count of *Staphylococcus aureus*

Out of 30 sample, *Staphylococcus aureus* presence in 12 samples. The maximum count was reported in Naikap i.e 6.8×10^4 then Bafal, Kalanki, Thamel and Sitapaila respectively. The maximum and minimum count of *Staphylococcus aureus* count was represented in Table 5.

Table 5: Location wise count of total *Staphylococcus aureus*

Location	**No of sample *Staphylococcus aureus***	**Maximum count**	**Minimum count**
Bafal	2	1.5×10^4	2×10^1
Kalanki	2	1.1×10^4	1×10^1
Naikap	4	6.8×10^4	1.1×10^2
Sitapaila	2	1.6×10^2	7.5×10^1
Thamel	2	3.3×10^2	2×10^1

4.1.5 Quality of paneer with reference to *Staphylococcus aureus* count

Out of 12 *Staphylococcus aureus* positive sample 6(50 %) samples had exceeded specification by FSSAI for *S.aureus*.The percentage of sample within maximum acceptable limit was in Sitapaila. Location wise distribution of quality of *Staphylococcus aureus* was represented on Table 5.

Table 5 :Location wise distribution of quality

Location	*S.aureus* +ve sample	Sample within acceptable level of <10 cfu/gm		Sample within marginally acceptable level of 10-100 cfu/gm		Sample exceeding marginally acceptable value <100 cfu/gm	
		N	%	N	%	N	%
Bafal	2	0	0	1	50	1	50
Kalanki	2	1	50	0	0	1	50
Naikap	4	0	0	1	25	3	75
Sitapaila	2	0	0	2	100	0	0
Thamel	2	1	50	0	0	1	50
Total	**12**						

4.1.6 Distribution quality of *Staphylococcus aureus* in samples

Out of 12 *Staphylococcus aureus* sample 3 samples had exceeded the specification for*S aureus*, according to acceptable level (>10^4) cfu/gm by ICMSF. Location wise distribution of quality of *Staphylococcus aureus* was represented on table 6.

Table 6: Location wise distribution of quality

Location	No of sample exceeding standard value >10^4 cfu/gm	cfu/gm
Bafal	1	1.5×10^4
Kalanki	-	-
Naikap	1	1.1×10^4
Sitapaila	-	-
Thamel	1	1.3×10^4

4.1.7 Antibiotic Susceptibility pattern of *Staphylococcus aureus* (n=12)

Out of 12 isolates of *S aureus*, 100% sensitive towards Gentamycin, Cotrimoxazole and Levofloxacin whereas resistance was observed in Ampicillin, Azithromycin, Cefoxitin, Chloramphenicol, Nalidixic acid, Penicillin G and Tetracycline. Antibiotic susceptibility pattern of *S aureus* are represented on Table 7.

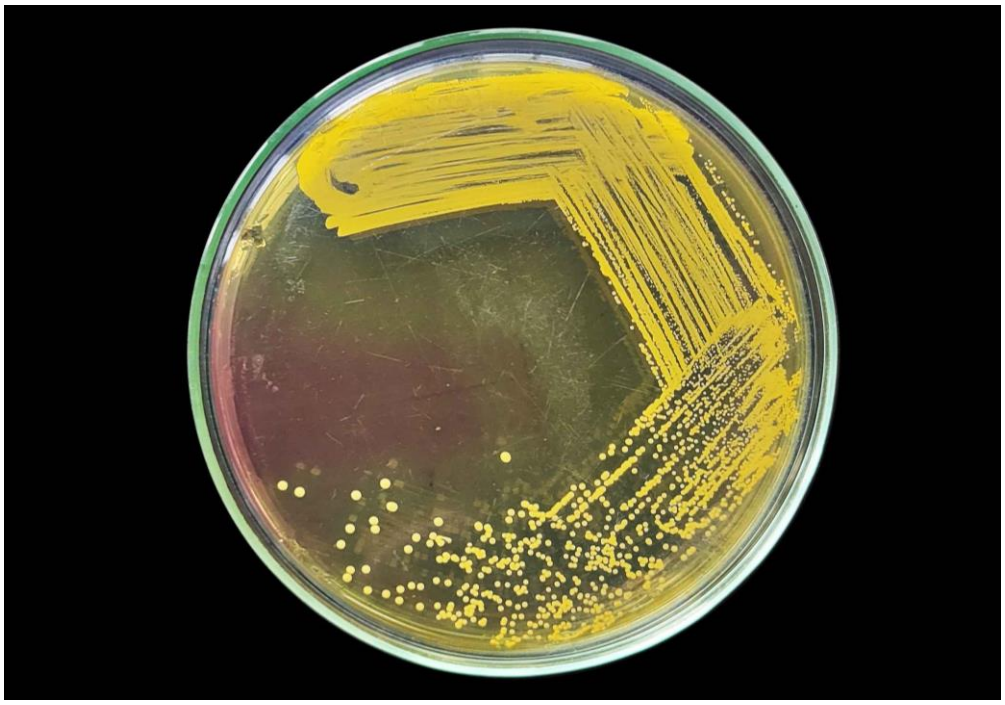
Table 7: Antibiotic Susceptibility pattern of *Staphylococcus aureus* (n=12)

Antibiotics	**Sensitive**		**Resistance**	
	n	**%**	**n**	**%**
Ampicillin(10mcg)	10	83.4	2	16.6
Azithromycin(15 mcg)	10	83.4	2	16.6
Cefoxitin (30mcg)	11	91.7	1	8.3
Chloramphenicol (30mcg)	11	91.7	1	8.3
Cotrimoxazole (25mcg)	12	100	0	0
Gentamycin (10mcg)	12	100	0	0
Levofloxacin (5 mcg)	12	100	0	0
Nalidixic Acid (30mcg)	7	58.4	5	41.6
Penicillin G (10mcg)	7	58.4	5	41.6
Tetracycline (30mcg)	11	91.7	1	8.3

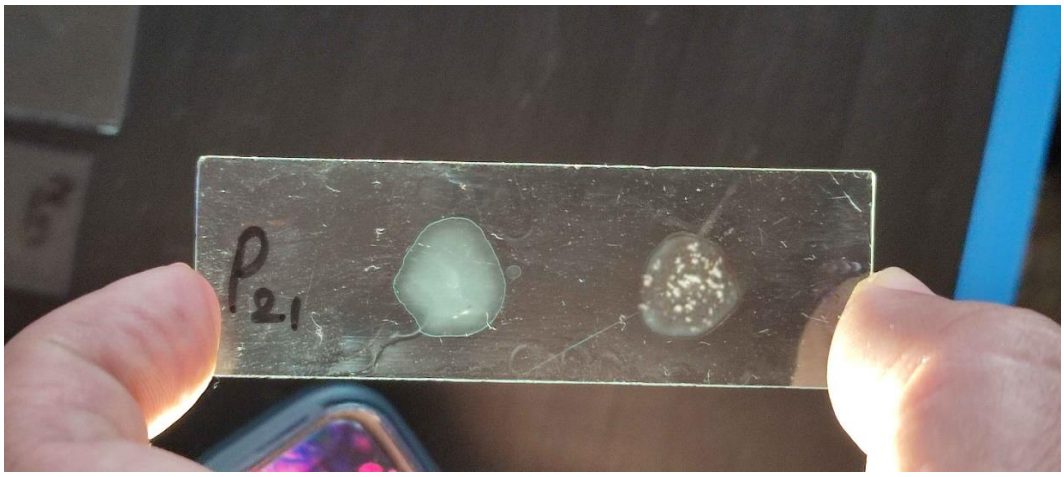
4.1.8 Detection of MRSA

Out of 12 isolates of *S.aureus* one isolate showed resistant towards Cefoxitin.According to susceptibility towards Cefoxitin showed 8.3% of *S. aureus* for dectection of MRSA .Only 1 isolates of paneer sample has shown resistance towards Cefoxitin. Therefore, Paneer sample from Naikap contain Methicillin resistant *Staphylococcus aureus* (MRSA).

PHOTOGRAPHS



Photograph 1: *Staphylococcus aureus* on Mannitol Salt Agar (MSA).

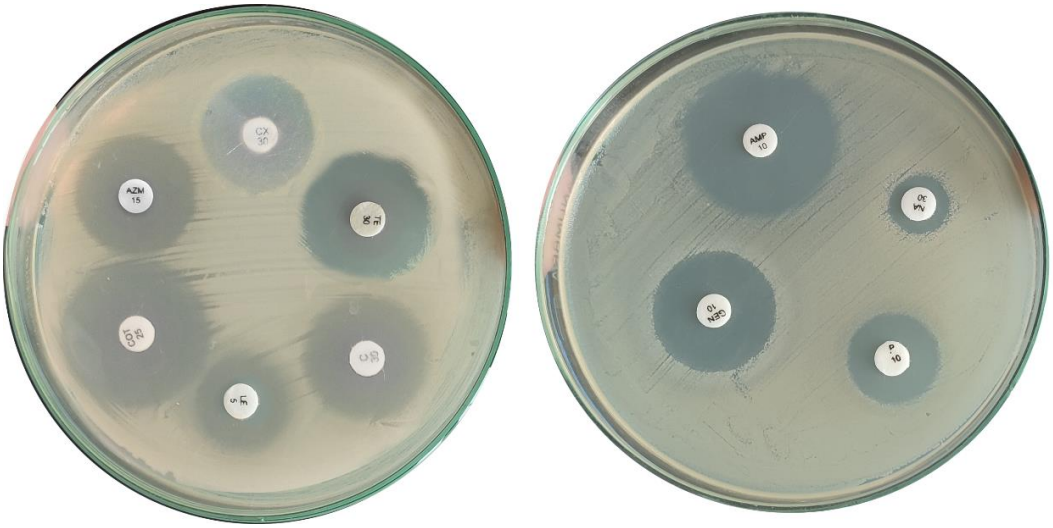


A B

Photograph 2: Slide Coagulase Test(A: Coagulase negative, B: Coagulase positive)

Fermentative

Photograph 3: Oxidative Fermentative test for *Staphylococcus aureus*



A B

Photograph 4: Antibiotic susceptibility test of *Staphylococcus aureus*
A=Cotrimoxazole (25mcg), Tetracycline (30mcg), Cefoxitin (30mcg) Chloramphenicol (30mcg), Levofloxacin (5mcg) Azithromycin (15 mcg)
B=Nalidixic Acid (30mcg), Gentamycin (10mcg), Ampicillin (10mcg), Penicillin G (10mcg)

Photograph 5: Sample processing in Microbiology Laboratory of Amrit Science Campus

4.2 DISCUSSION

The poor bacteriological quality of market paneer was mainly due to the use of poor quality milk, unhygienic practices during manufacturing handling and storage of product (Singh and Singh 2000). Presence of pathogenic microorganism in some significant number can cause foodborne diseases. Milk and milk product may be contaminated with a wide range of potentially harmful chemicals. These compounds enter milk through various direct and indirect routs (Stadler *et al*. 2011).

In the microbial evaluation of 30 paneer samples, maximum total bacterial count was found in Naikap i.e. 9×10^6cfu/gm with an average count 3.1×10^6cfu/gm. This may be due to contamination from different source including the poor quality of raw milk and processing in uncontrolled environments (Sameh, 2016).In similar study carried out by Grove *et al.*2001 viable bacterial counts obtained ranged from 3×10^2 to 9.7×10^{10} CFU/mL. Contamination of paneer by pathogenic bacteria could be an important factor of gastrointestinal illnesses in the consumers (Grove, *et al.*,2001).

In this study, among 30 samples, 39.9% sample were exceeded the specification value of total bacterial count. High bacterial growth in milk products due to directly enter from milk and poor productions process.

In 30 sample, maximum *Staphylococcus spp.* count was found in Naikap i.e 8×10^5cfu/gm with an average 4×10^5cfu/gm. Noor *et al.* 2011 studied maximum *Staphylococcus spp* count was found 4.7×10^7 cfu/ml. Similarly Simkhada,*et al.* (2019) reported the maximum *staphylococcus* count was 2.1×10^4 cfu/gm. The high Staplylococcal count may be due to contamination of milk, disease such as mastitis in cattle etc. *Staphylococcus spp.* can be attributed to poor hygienic conditions during paneer preparation, handling and storage (Godbole *et al.*,2013).

In this study, among 30 paneer sample,40.2% samples were identified as *S aureus* and 56.9% samples were other Staphylococci. Similarly Simkhada,*et al.* (2019) reported 20.8% were *S.aureus* and 79.2% were other Staphylococci.

In our study among the 30 paneer samples, 40.2% (12) sample were contaminated with *S. aureus* Mukadderat *et al*.(2013) from Turkey, Girdharwal(2018) from India and Ekici *et al.*(2018) from Pakistan reported 13.0% of cheese samples, 27.3% paneer samples a

samples were contaminated with *S. aureus*. Similarly Simkhada, *et al.*(2019) from Kathmandu, reported 20.8% samples were contaminated with *S. aureus* . Compared to study carried out by Simkhada, *et al.*(2019) percentage of contamination by *S. aureus* was lower in our study. Presence of high number of *S.aureus* in milk product may be due to directly enter from milk and poor hygiene of milkers, utensils and milk handlers, Udder of cow. Expressed breast milk contains bacteria, which inhibit *Staphylococcus aureus*. *Staphylococcus aureus* can be effectively killed by pasteurization but the enterotoxin produce by *S. aureus* retain their biological activity even after pasteurization, which become hazard for consumers (Asao, *et al.*,2003). Toxins released by mastitis bacteria damage milk secreting tissue and ducts throughout the mammery gland, reducing milk yield and quality. A major causative pathogen is *S.aureus* which can contaminate milk from sick cows or from handlers.

In our study among the 30 paneer samples, 20% (6) sample exceeded the specification value of *Staphylococcus aureus* given by FSSAI which is >100 cfu/gm. In the study done by Sonune (2018) reported 40% (80) samples was contaminated with *S aureus* and 60% of sample did not conform to guideline value. Detection of high *S.aureus* count in milk products indicates the danger of food intoxication, as strain of *S.aureus* could produce enterotoxins A,B,C,D and E under favorable conditions (Thaker, *et al.*,2013).

In this study 10% samples exceeded the $\leq 10^4$ cfu/gm of *Staphylococcus aureus* given by ICMSF. However Simkhada, *et al.*(2019) reported 28.6% samples exceeded guideline value. In this study of 90% samples were within the is $\leq 10^4$ cfu/gm of *S. aureus* which is higher than reported by Simkhada.The difference in the prevalence of *S. aureus* in dairy sample may be linked with quality of milk origin from the milking process, insufficient pasteurization, and post pasteurization process like manufacture, storage and handling (Thaker,*et al.*, 2013). *S. aureus* when ranges 10^4-10^6 cfu/gm, Staphylococcal enterotoxins were produced which causes food intoxication (Seo, *et al.*, 2007). These samples with toxins and *S. aureus* on consumption could cause several foodborne diseases and food intoxication as researchers have indicated that even 0.5 ng/gm level of toxins can cause foodborne intoxication (Kadariya, *et al.*, 2014).

Antimicrobial susceptibility testing of *S. aureus* from paneer sample in this study has shown 100% sensitivity towards Co-trimoxazole, Gentamicine and Levofloxacin.There was reduced susceptible of *S.aureus* towards Tetracycline (91.66%), Cefoxitin

(91.66%),Penicillin (58.3%), Chloramphenicol (91.66%), Penicillin G (58.33%), Nalidixic Acid (58.33), Azithromicin (83.3%) and Ampicillin (83.3%) According to Nijom, *et al.* Antibiotic susceptibility of *S. aureus* from paneer sample revealed varying degrees of susceptibility patterns against the antimicrobial agents. Generally Cefoxitin 76.7%, Chloramphenicol 83.3%, levofloxacin 86.7% were the most effective antibiotics to *S.aureus*. Matallah, *et al.*(2019) observed that *S. aureus* was 49.7% resistance to penicillin G,%.3% to Tetracycline, 2.1% to Cefoxitin and 100 % sensitive to Gentamycin and Chloramphenicol.

In this study, the isolated *Staphylococcus aureus* mostly resistance to Cotrimoxazole, Gentamycin and Levofloxacin and sensistive to Nalidixic acid,Cefoxitin,Chloramphenicol,Tetracycline,Azethromycin,Ampicillin and Penicillin G. NCRP (2019) studied that the isolated *Staphylococcus aureus* mostly resistance to Ampicillin and sensitive to Tetracyclines followed by Cholramphenicol, Gentamycin. Simkhada, *et al.* (2019) studied the antimicrobial susceptibility pattern of *S. aureus* showed 100% sensitivity towards Chloramphenicol, Cotrimoxazole, Gentamycin, Vancomycin and Tetracycline whereas resistance towards Penicillin G, Ampicillin, Nalidixic acid.

An increasing number of people are being diagnosed with skin infections caused by *Staphylococcus aureus* bacteria that are resistance to many antibiotics.These resistance strains of *Staphylococcus* are MRSA. Staphylococcus species are developing resistant to different antibiotics day by day. Number of factors contribute to increase in the rate of resistance against antibiotics and some of those are poor regulations regarding antibiotics, easy and over the counter availability without prescription, unqualified personnel.

In this study, Methicillin resistant*S. aureus* was also isolated from paneer sample. 1 (3.33%) *Staphylococcus* species was identified as MRSA. Simkhada, *et al.*2019 found that among *S. aureus* 19.0% isolates were Methicillin resistance *S aureus*. Similarly, Joshi, *et al.*.(2014), Haren, *et al.*,(2012) and Juhasz-Kaszanyitzky, *et al.*(2007) reported 11.3%, 4% and 1.4% of MRSA in paneer sample respectively. It has been documented that human develop drug resistant bacteria such as Staphylococcus from food of animal origin (Landers, *et al.*,2012).examples of drugs that have been shown to cause the growth of resistant bacteria in foods from animals are fluoroquinolones and avaoparin (NRC, 1991)

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study, maximum count of total bacteria was found in Naikap where as minimum count was in Sitapaila. Maximum number of samples from Sitapaila was within acceptable guideline according FSSAI requirements. In this investigation, 39.9% paneer sample exceeded acceptable limit of total bacteria from total sample and 20% paneer sample exceeded acceptable limit of *Staphylococcus aureus* from total sample. This study concluded that the high number of bacteria were detected in Naikap area and sample from Naikap showed higher antibiotic resistance compared to other areas. Therefore, the presence of *S. aureus* and total bacteria above the unacceptable limits in paneer sold in Kathmandu constitutes a major health risk. According to findings of this study, monitoring susceptibility pattern of *Staphylococcus aureus* is essential for tracking the emergence of antibiotic resistance. Prior to commercialization of milk products such as paneer microbiological research is necessary for risk assessments.

5.2 Novelty and National Prosperity aspect of Project work

Paneer is indigenous dairy products, which is made from milk. It is consumed by all age group of people**.** Paneer is integral part of a significant population of Nepal. Milk production in Nepal not same all year round but the demand for milk products in the markets also have fluctuations. Milk product diversification as well as maintenance of quality and development of dairy products such as paneer is essential where surpluses milk in milk holidays, lockdown and strikes could be used. The data of antibiotic susceptibly of testing of this project work can be used in suggestion of the effectiveness of the respective drugs. Establishment of national standard microbial guideline for paneer and continuous microbial quality monitoring is essential. This will help in improving the public health aspect at the national level.

5.3 Limitation of Study

This project was completed in the Amrit Campus laboratory. Sample size in this study was small, which restrict to find significant relationships from the data. Time was also restricted for study so physical quality analysis of Paneer could not be done. Investigation of the source of contamination and other factors such as moisture, pH, water activity was not done due to restriction of time.

Recommendation

1. Milk should be collected in clean equipment from healthy animals and also be properly pasteurized for the production of Paneer.

2. Proper packaging should be implemented to minimize product loss.

3. For Microbiological quality monitoring for Paneer Nepal Standard should be made.

4. Large scale microbiological quality monitoring of marketed paneer should be done regularly by authorized Department/ Institution of Government Nepal for quality control.

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APPENDIXES

Appendix I:

List of materials

Equipment

1. Autoclave (Life, India)
2. Incubator (Leader, UK and Memmert, Germany)
3. Hot air oven (Ambassador, India)
4. Electronic balance (Phoenix instrument, Germany)
5. Refrigerators (LG and Whirlpool, India)
6. Binocular microscope (COSLAB, India)
7. Leminar air flow (ACCO,India)
8. Bunsen Burner
9. Icebox (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

Chemical and reagent

1. Crystal Violet
2. Gram's Iodine
3. Acetone - Alcohol
4. Safranine
5. 3% Hydrogen peroxide solution

6. 1% Tetramethyl p–phenylin-diaminedihydrochloride
7. 1N Hydrochloric acid (HCL)
8. Paraffin oil
9. Normal saline

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

1. Mannitol Salt Agar
2. Nutrient Agar
3. Nutrient Broth
4. Muller Hinton Agar
5. Hugh and Leifson's Media
6. Plasma

Appendix II:

Composition and preparation of sample diluent

Normal Saline Ingredients	**Amount**
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 100 mL distilled water was added to a beaker and dissolved completely and transferred to 1 litre volumetric flask. Then, distilled water was added upto 1 litre mark, and mixed well. Then the solution was dispensed on a testtube and conical flask and then sterilize by autoclaving at 15 pressure for (121^{o}C) 15 minutes.

Appendix III:

Composition and preparation of staining reagents

Gram staining reagents

Crystal Violet

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

Preparation:

In a piece of clean paper, 20 gm 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

Composition	**Amount**
Potassium Iodide	2 gm
Iodine	1 gm
Distilled Water (D/W)	100 mL

Preparation: 2 gm of Potassium Iodide was taken and transferred to a brown bottle premarked to hold 100 mL. Then about 30 mL distilled water was added and mixed well until the Potassium Iodine was dissolved completely. Then, 1 gm iodine was added to Potassium Iodide solution and mix well until the iodine was dissolved completely. Then the solution was make upto 100 mL distilled water and mixed well.

Acetone-Alcohol

Composition Amount

Acetone 500 mL

Ethanol (absolute) 475 mL

Distilled Water (D/W) 25 mL

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

Safranine

Composition	**Amount**
Safranine (2.5% solution in 95% ethanol)	10.0 mL
Distilled Water (D/W)	100 mL

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

Appendix IV:

Composition and preparation of microbiological media

Manitol Salt Agar

Ingrediant	**Gms/Litre**
Proteose peptone	10.000
HM Peptone B#	1.000
Sodium chloride	75.000
D-Manitol	10.000
Phenol red	0.025
Agar	15.000
Final pH (at 25^0)	7.4±0.2

Preparation: 111 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely, it was sterilized by autoclaving at 15 psi pressure (121^0C) for 15 minutes.

Nutrient Agar (NA)

Ingredients	**Amount (gm/L)**
Peptone	5.00
Sodium Chloride	5.00
HM peptone B #	1.50
Yeast Extract	1.50
Agar	15.00

Final pH (at 25^{o}C) 7.4 ± 0.2.

Preparation: 28 gm of the media was suspended in 1000 mL distilled water and boiled to dissolve completely then sterilized by autoclaving at 15 psi pressure (121^{o}C) for 15 minutes and then poured 18-20 mL into sterile petriplates at around 45°C temperature. Then the media was cooled to around 45°C and poured to sterilized 90 mm petriplate around 18-20 mL.

Nutrient Broth (NB)

Ingredients	**Amount (gm/L)**
Peptic digest of animal tissue	5.00
Yeast Extract	1.50
Beef Extract	1.50
NaCl	5.00

Final pH (at 25°C) 7.4 ± 0.2

Preparation: 25 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 psi pressure (121°C) for 15 minutes.

Muller Hinton Agar (MHA)

Ingredients	**Amount (gm/L)**
H Minfusion B from	300.00
Acicase	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 psi pressure (121^{o}C) for 15 minutes. Then the media was cooled to around 45°C and poured to sterilized 90 mm petriplate around 18-20 mL.

(Hugh Leifson's) Basal Media

Ingredients	**Amount (gm/L)**
Casein enzymic hydrolysate	2.00
NaCl	5.00
Dipotassium Phosphate	0.30
Bromothymol blue	0.08
Agar	2.00

Final pH (at 25°C) 6.8 ± 0.2

Preparation (100 mL): 0.938 gm of Hugh Leifson's media and 2 gm 1% dextrose was suspended in 100 mL distilled water and boiled to dissolve completely. The media was dispensed in test tubes about 4-5 mL and sterilized by autoclaving at 10 psi pressure (110°C) for 15 minutes.

Appendix V:

Composition and preparation of biochemical reagents

Catalase Reagent (3% H_2O_2)

Composition	**Amount**
Hydrogen Peroxide solution (6% H_2O_2)	10 mL
Distilled water (D/W)	90 mL

Preparation: To 90 mL distilled water, 10 mL of hydrogen peroxide was added and mixed well.

Oxidase Reagent

Ingredients	**Amount**
Tetra methyl-p- phenyl diamine dihydrochloride (TPD)	1.00 gm
Distilled water (D/W)	100 mL

Preparation: This reagent solution was made by dissolving 1 gm TPD in 100mL distilled water. The strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

Hydrochloric acid (1 N HCL)

Ingredients	**Amount**
Hydrochloric acid (1 mol/L)	8.6 mL
Distilled water (D/W)	91.4 mL

Preparation (100 mL): About 50 mL distilled water was added to a 100 mL volumetric flask. Then 8.6 mL hydrochloric acid (1 mol/L) was added and make volume upto 100 mL mark by adding distilled water. Then solution was mixed well.

Appendix VI:

Principle and procedure for Gram staining and biochemical properties

Gram staining:

Principle of Gram staining:-

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 satined by safranine (appear red/pink) are referred as Gram negative.

Procedure for Gram staining: -

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.

The oxidase test is used to assist in the identification of *Pseudomonas, Neisseria, Vibrio, Brucella,* and *Pasteureella* species, all of which produce the enzyme cytochrome c oxidase.

Procedure:-

1.A piece of oxidase paper was taken on clean glass slide (filter paper soaked with the oxidase reagent 1% tetra methyl- p- phenyl 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 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°C for 24-48 hrs and observed for color change in both the tubes.

Coagulase test

Principle:

The coagulase test identifies whether an organism produces the exoenzyme coagulase, which causes the fibrin of blood plasma to clot.

Slide coagulase test

Slide coagulase test bound coagulase (clumping factor) is detected by slide test results in alteration of fibrogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell,causing the cells to clump when a bacterial suspension is mixed with plasma.

Procedure

1. A drop of physiological saline was placed on two places of slide and then a colony of the test organism was emsulfied in two of the drops to make thick suspension.
2. It was then, a drop of plasma was added to one of the suspensions and mixed gently
3. Then a clumping was observed within 10 seconds for the positive coagulase test.
4. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping.

Source: Cheesebrough (2006)

APPENDIX VII:

Method of quality evaluation

Enumeration of total bacteria

The test sample was prepared in accordance with the relevant part of ISO 6887. Paneer sample (10 gm) was homogenized aseptically in 90ml 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 9mL sterile normal saline and similarly diluted upto 10^{-6}. About 15 ml of the plate count agar at 44 °C was poured into each Petri dish. Then inoculum with the medium was mixed by rotating the Petri dishes and allow themixture to solidify by leaving the Petri dishes standing on a cool horizontal surfaceand incubated at 37^0C for 24 to 48 hours**.** It was then, total bacteria were counted.

$$\text{Cfu/gm} = \frac{\text{No. of colonies x Dilution factor}}{\text{Volume of sample taken}}$$

Preparation sample and enumeration of *Staphylococcus* spp

Paneer sample (10 gm) was homogenized aseptically in 90ml 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 9mL sterile normal saline and similarly diluted upto 10^{-6}. Then, 0.1 mL of diluted sample from each dilution was transferred onto Manitol Salt agar (MSA) and spread using sterile bent L- shaped glass rod and incubated at 37^0C for 24 to 48 hours. After that, enumeration of isolated colonies and calculation of *S. aureus* (golden yellow colonies) in paneer sample were done by using colony forming unit (cfu/gm) formulae (BAM online 2001, Gomez *et al.*,2018, ISO 2012)

$$\text{Cfu/gm} = \frac{\text{No. of colonies x Dilution factor}}{\text{Volume of sample taken}}$$

Antibiotic susceptibility testing

The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. The most widely used testing method includes broth microdilution or rapid automated instrument methods that use commercially marketed materials and devices(Reller *et al.*,2009).

There are a number of different method of AST such as agar dilution, broth dilution and disc diffusion assays.The culture of standard bacteria, American Type Culture Collection(ATCC), has been used for decades as a control reference, some of which are mandated or recommended in both FDA (2012) regulation and in the standard put out in Clinical and Laboratory Standards Institute (CLSI) (Simione 2011).

Kirby-Bauer disc diffusion method

The disc diffusion or Kirby- Bauer method involves spreading bacteria on an agar plate and placing paper disc impregnated with antibiotic on the plate.After incubation the growth of bacteria ia observed. Areas around the antibiotic disc where bacterial growth can be seen are known as ‘zone of inhibition’.These zones show that an antibiotic has been successful in stopping bacterial growth or killing the bacteria. By measuring the diameter of these zones,we can compare the efficacy of antibiotics and monitor antimicrobial resistance.

Antibiotic Susceptibility Testing

Procedure

1.Using a sterile inoculating loop, single isolated colony of the test organism was inoculated into 2-3 mL of nutrient broth and was incubated at 37°C for 4 hours.

2.Then, the turbidity of the prepared inoculum was compared and adjusted with the MacFarland turbidity standard no. 0.5.

3.Then, sterile cotton swab was dipped into the nutrient broth containing inoculum. Excess inoculum was removed by pressing and rotating the swab against the side of the tube wall above the level of suspension.

4. Then, the inoculum was swabbed evenly over the surface of the MHA media in three directions, rotating the plate approximately 60° to ensure even distribution to obtain lawn culture.

5. With the petri-dish lid close, the surface of the agar was allowed to dry for 3-5 minutes.

6. Then, the antibiotics were placed on the surface of agar with the help of sterile forceps and pressed gently.

7. Within 30 minutes of applying the discs, the plate was inverted and then it was incubated at 35 ± 0.2^{o}C for 16-18 hours.

8. The susceptibility pattern was noted following overnight incubation by measuring zone of inhibition as sensitive, intermediate and resistant as according to zone size interpretative chart.

APPENDIX VIII;

Preparation of Mc Farland

Mc Farland 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 McFarland standard no. 0.5

1. 1% v/v solution of Sulphuric acid as prepared by adding 1mL of concentrated sulphuric acid to 99 mL water.
2. 1% w/v solution of barium chloride was prepared by dissolving 0.5gm of dehydrate barium chloride ($BaCl_2.2H_2O$) in 50 mL of distilled water.
3. 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 turbidity standard, equivalent to a 0.5 McFarland standard was used.

A: Zone size interpretative chart

For *Staphylococcus aureus*,
Source: CLSI (2013, CLSI 2018)

Antibiotic Disc	Code	Disc content (mcg)	Sensitive	Intermediate	Resistance
Tetracycline	Te	30	19	15-18	14
Penicillin G	P	10	29	-	28
Cefoxitin	CX	30	22	-	21
Choramphenicol	C	30	18	13-17	13
Nalidixic acid	NA	30	20	19	14-18
Azithromycin	AZM	15	18	14-17	13
Levofloxacin	LE	5	17	14-16	13
Co-Trimoxazole	COT	25	17	14-16	14
Gentamicin	GEN	10	18	-	18
Ampicillin	AMP	10	29	-	28

Appendix IX:

Morphological and cultural characteristics of *Staphylococcus aureus*

BACTERIA	MORPHOLOGICAL CHARACTERISTICS	CULTURAL CHARACTERISTICS
Staphylococcus aureus	Gram positive,non motile,catalase positive, small,spherical bacteria (cocci),appear in pairs,short chains or bunched in grape like cluster.	Manitol Fermenter,smooth and glistening yellow colonies on Manitol Salt Agar.

Source: FDA (2012) Cheesebrough (2006)

Appendix X:

Biochemical tests for identification of bacteria

Organism	Test			
	Catalase test	Oxidase test	Oxidative fermentative test	Coagulase test
Staphylococcus aureus	+	_	F	+

Appendix XI:

Total Plate count and Total *Staphylococcus spp* Count of Paneer

Sample code	Total Bacterial count (cfu/gm)	Total Staphylococcal Count (cfu/gm)
P_1	1.8×10^4	1.1×10^2
P_2	9×10^6	7×10^3
P_3	4×10^5	3×10^1
P_4	1.1×10^5	1×10^3
P_5	1×10^6	11.5×10^3
P_6	1.3×10^3	1.6×10^2
P_7	1.3×10^3	10
P_8	5×10^5	15.1×10^2
P_9	1.7×10^4	4.4×10^2
P_{10}	0	0
P_{11}	1.3×10^5	68.6×10^3
P_{12}	2×10^4	1.3×10^3
P_{13}	4.5×10^4	2×10^1
P_{14}	5.9×10^5	2.3×10^2
P_{15}	6.2×10^3	2.3×10^2
P_{16}	6.8×10^2	1.2×10^2
P_{17}	3×10^6	15.3×10^3
P_{18}	6.9×10^4	2.1×10^2
P_{19}	4×10^5	8×10^1
P_{20}	2×10^1	1.7×10^1
P_{21}	3.2×10^4	4×10^1
P_{22}	2.3×10^4	7.5×10^1
P_{23}	62×10^1	1.6×10^2
P_{24}	1.4×10^3	2×10^1
P_{25}	1.3×10^5	6×10^1
P_{26}	1.9×10^2	1.7×10^2
P_{27}	3.5×10^3	2×10^1
P_{28}	6.4×10^4	1.5×10^2
P_{29}	8×10^6	-
P_{30}	7.4×10^6	3.3×10^2