MICROBIOLOGICAL QUALITY AND ADULTERATION OF PASTEURIZED MILK MARKETED IN DHARAN, NEPAL

A

Project work submitted to

Department of Microbiology

Central Campus of Technology, Tribhuvan University

In Partial Fulfillment for the Award of the Degree of

Bachelor of Science in Microbiology

Submitted by:

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RECOMMENDATION

This is to certify that **Ms. Monika Ghimire** has completed this project work entitled **"Microbiological Quality and Adulteration of Pasteurized Milk Marketed in Dharan, Nepal."** as a part of partial fulfillment of the requirements of B.Sc. degree in Microbiology under our supervision. To our knowledge this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Dhiren Subba Limbu,** this project work of **Ms. Monika Ghimire** entitled **"Microbiological Quality and Adulteration of Pasteurized Milk Marketed in Dharan, Nepal."** has been approved for the examination and is submitted to the Tribhuvan University in Partial fulfilment of the requirements for B.Sc. degree in Microbiology.

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ABSTRACT

This study was aimed to evaluate the quality of pasteurized milk and also compare among different brands of pasteurized milk marketed in Dharan. Milk may be contaminated with pathogenic microorganisms and a mixture of several adulterants and such milk pose a risk to consumers. The study was carried out from September 2019 to January 2020. Collected samples were tested for adulterants (starch, formalin, neutralizer and table sugar) as well as microbial analysis (Total Coliform count, Total Viable Count, Thermoduric Count, *Escherichia. coli* and *Staphylococcus aureus*) as per standard guideline. The adulterants starch, formalin and neutralizer were not detected. However, table sugar was present in 90% (18 out of 20) pasteurized milk.

The average Total Viable Count, Total Coliform Count and Thermoduric Count of pasteurized milk were found to be 15×104 CFU/ml, 14×103CFU/ml and 4×103 CFU/ml respectively. *E. coli* was detected in 30% pasteurized milk whereas *S. aureus* was isolated from only 20%.

The results of the study indicated that routine monitoring of dairy industries awareness campaign and good hygienic practice should be promoted to upgrade the quality of pasteurized milk.

Key words: Milk adulteration, Defective pasteurization, Milk handlers, Chemical adulterants.

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ABBREVIATIONS

CFU/ml Colony forming unit per millilitre

TPC Total Plate Count

TVBC Total Viable Bacterial Count

TSC Total Staphylococcal Count

TCC Total Coliform Count

TB Thermoduric Bacteria

PHE Plate Heat Exchanger

DFTQC Department of Food Technology and Quality Control

DDC Dairy Development Corporation

N MC Nepal Multipurpose Co-operative

NDDB Nepal Dairy Development Board

SNF Solid Not Fat

EU European Union

MNS Mandatory Nepalese Standard

ND Not Detected

GMP Good Manufacturing Practice

SSOP Sanitation Standard Operating Procedure

HACCP Hazard Analysis and Critical Control Point

PCA Plate Count Agar

CCT Central Campus of Technology

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CHAPTER I
INTRODUCTION

1.1 Background

Milk is the lacteal secretion of the mammary glands of a mammal. It is the first natural food of all young mammals during the period immediately after birth (Reta, 2015). Milk is a complete diet containing all essential nutritional constituents (Baharullah Khattak et al., 2013). Buffalo milk contains 7.6% fat, 3.8% protein, 4.9% lactose, 0.78% ash and 17% total solids. Cow milk contains 4.5% fat, 3.8% protein, 4.9% lactose, 0.72% ash 13.9% total solids. That is why milk is an important part of the diet of all age group including expectant mothers (wijesinha-bettoni & Burlingan, 2013). Milk and dairy foods are nutrient-dense foods supplying energy and significant amounts of protein and micronutrients. The inclusion of dairy products adds diversity to plant-based diets. (Dhamala, 2018). Milk can be regarded as a complete food, containing protein, fat, lactose, vitamins and minerals, together with natural enzymes and those derived from microorganisms within the milk (Dhamala, 2018). Milk and milk products are highly susceptible to microbial contamination because their composition provides a favourable medium for the growth of a wide variety of microorganisms (De Buyser, Dufour, Maire, & Lafarge, 2001). When asceptically drawn, milk is sterile; however, it is contaminated during and after secretion and during the normal process of production and procession. Infection of the mammary gland, udder and teat surfaces, milking equipment's and storing tanks all have the potential to contaminate milk (Gleeson, O'Connell, & Jordan, 2013). Milk is a nutritious medium that can support the growth of a large selection of bacterial contaminants. Bacteria are capable of utilizing the proteins, fats, carbohydrates and vitamins in milk for their growth and metabolism (Gleeson et al., 2013). Contamination of milk may occur through various sources. May be through infected cow with tuberculosis, brucellosis, and mastitis and also from milk handlers infected with typhoid fever, diphtheria, dysentery, and scarlet fever (Jay et al., 2005). It is common that dairy cattle and their farm's surroundings may contain many pathogens such as *Listeria, Salmonella,* and pathogenic *E. coli.* Raw or inadequately pasteurized milk may contain toxin producing *E. coli, Salmonella , Listeria monocytogenes* and others (Pal et al., 2016). Poor pre-milking udder hygiene that fails adequately to clean dirty udders may also result in the introduction of vegetation, soil, and bedding material and their associated

microorganisms into the milk, such foreign matters and contaminations in the milk may lead to concerns regarding consumer health. Hence the safety of milk and its products is of great concern around the world. This is even bigger concern in developing and under developed nation where milk and its products are prepared in unsanitary conditions (Dhungel;, Maskey;, Bhattarai;, & Shrestha;, 2019). To protect public health against food borne infections, there are regulations that require handling of milk and its pasteurization, but in developing countries such regulations are not usually adhered, hence milk borne health risk is higher in these countries (Rahmatalla, Elsheikh, & Abdalla, 2016). Presence of *S. aureus* and an intestinal commensal *E. coli* indicates the alarming public health concern. To minimize the risk of milk-borne diseases, an intense study should be done to determine the microbiological quality of milk and other chemical adulterants and their public health impact (Arjyal et al., 2004). Thus, objectives of this study were to determine the bacterial contaminants as well as an admixture of adulterants in pasteurized milk marketed in Dharan.

1.2 Significance of the study

This study provides an information of microbiological quality and use of adulterants in local market milk in Dharan area. And also provides an information on impacts of microorganism and adulterants in human body and effects of pasteurization in microorganism contaminating milk.

1.3 Objectives of the study

1.3.1 General objective

To study the microbiological quality and adulteration of pasteurized milk marketed in Dharan.

1.3.2 Specific objectives

a) To isolate and identify *S. aureus* and *E. coli* from different milk samples.
b) To enumerate the organisms by total plate count method, total coliform count and total staphylococcal count.
c) To test the presence of adulterants starch, formalin, sugar and neutralizer in pasteurized milk.
d) To study about total microbial quality of pasteurized milk marketed in Dharan.

1.4 Limitations of the work

1. Shelf life of the product was not studied due to time constraint.
2. Melamine, urea, vegetable or animal fat, whey protein was not tested.

CHAPTER II

LITERATURE REVIEW

2.1 Background

Milk has been referred to as the "most nearly perfect" food for man. Its nutritive value is reflected by its chief constituents namely, proteins, carbohydrates, fats, minerals, vitamins and water (Pelczar MJJR, Chan ECS, & NR, 1993). The chief constituents of milk are approximately as follows:

Table I: Composition of milk

Constituents	Percentage (%)
Water	87.25
Dry matter	12.75
Fat	3.80
Protein	3.50
Sugar	4.80
Ash	0.65

Source: Eckles C.H.

Milk in its natural state is a highly perishable material because it is susceptible to rapid spoilage by the action of naturally occurring enzymes and contaminating microorganisms *E. coli, Staphylococcus spp., Lactobacillus, Bacillus spp., Streptococcus spp., Listeria monocytogenes* etc. are the pathogens commonly found in milk. Among them *E. coli* and *S. aureus* are the most common contaminants. These microorganisms may gain access to milk or the products of the milk through: (1) interior of the udder, (2) exterior of the cow's body, (3) atmosphere, (4) utensils, (5) milker or handler, and (6) various ingredients added to dairy products (Eckles CH, Combs WB, & H, 1951). Fresh milk drawn from a healthy cow normally contains a low microbial load (less than 1000 per ml), but the number may increase up to 100-fold or more once it is stored for sometimes at normal temperature (Richter et al 1992). However, keeping milk in clean containers at refrigerated temperatures immediately after milking process may delay the increase of microbial load and prevent the multiplication of microorganisms in milk between milking at the farm and transportation to the processing plant (Adesiyun, 1994).

2.2 Pasteurization

The history of pasteurisation is documented in Cronshaw (1947) and this makes interesting reading. In fact the first stage in the history of pasteurisation between 1857 and the end of the nineteenth century might well be called the medical stage (smit, 2003).

The first positive Holder pasteurisation system was introduced in Germany in 1895 and in the USA in 1907. Thus by 1895 what was required for an effective pasteurisation process was well recognised: 'we know that this process (pasteurisation) if properly carried out will destroy all disease germs' and 'a thoroughly satisfactory product can only be secured where a definite quantity of milk is heated for a definite period of time at a definite temperature(smit, 2003). Pasteurization is a process, named after scientist Louis Pasteur that applies heat to destroy human pathogens in food. Pasteurization is defined as the process of heating every particle of milk or milk product in properly designed and operated equipment to a specific temperature for a specified period of time. It makes the milk safe and healthy and also helps to prolong its shelf life (Tessema: & Tibbo, 2009). Pasteurization kills milk borne pathogens responsible for diseases such as Listeriosis, Typhoid fever, Tuberculosis, Diphtheria and brucellosis; it also kills the harmful bacteria *Salmonella, S. aureus, Yersinia, E. coli 0157:H7, Campylobacter* (Smith, 1981).

Table II: Heat treatments of milk

Process	**Temperature**	**Time**
Thermisation	63 – 65	15 s
LTLT pasteurization of milk	63	30 min
HTST pasteurization of milk	72-75	20 sec
Ultra-pasteurization	125-138	2-4 sec
UHT (flow sterilisation)	135-140	2/3 sec
Sterilisation in container	115-120	20-30 min

(Gösta Bylund, 2003)

2.2.1 Thermisation

In many large dairies, it is not possible to pasteurise and process all the milk immediately after reception. Some of the milk must be stored in silo tanks for hours or days. Under these conditions, even deep chilling is not enough to prevent serious quality deterioration. Many dairies therefore pre-heat the milk to a temperature below the pasteurisation temperature, to temporarily inhibit bacterial growth. This process is called thermisation. The milk is heated to 63 – 65 °C for about 15 seconds, a time/temperature combination that does not inactivate the phosphatase enzyme. Double pasteurisation is forbidden by law in many countries, so thermisation must stop short of pasteurisation conditions. To prevent aerobic spore-forming bacteria from multiplying after thermisation, the milk must

be rapidly chilled to 4 °C or below and it must not be mixed with untreated milk. Many experts are of the opinion that thermisation has a favourable effect on certain spore-forming bacteria. The heat treatment causes many spores to revert to the vegetative state, which means that they are destroyed when the milk is subsequently pasteurized (Gösta Bylund, 2003).

2.2.2 LTLT pasteurisation

The original type of heat treatment was a batch process in which milk was heated to 63 °C in open vats and held at that temperature for 30 minutes. This method is called the holder method or low temperature, long time (LTLT) method. Nowadays milk is almost always heat treated in continuous processes like thermisation, HTST pasteurisation or UHT treatment (Gösta Bylund, 2003).

2.2.3 HTST pasteurisation

HTST is the abbreviation of High Temperature Short Time. The actual time/temperature combination varies according to the quality of the raw milk, the type of product treated, and the required keeping properties (Gösta Bylund, 2003).

2.2.3.1 Plate heat exchangers

Most heat treatment of dairy products is carried out in plate heat exchangers. The plate heat exchanger (often abbreviated PHE) consists of a pack of stainless-steel plates clamped in a frame. The frame may contain several separate plate packs – sections – in which different stages of treatment, such as pre-heating, final heating and cooling take place.

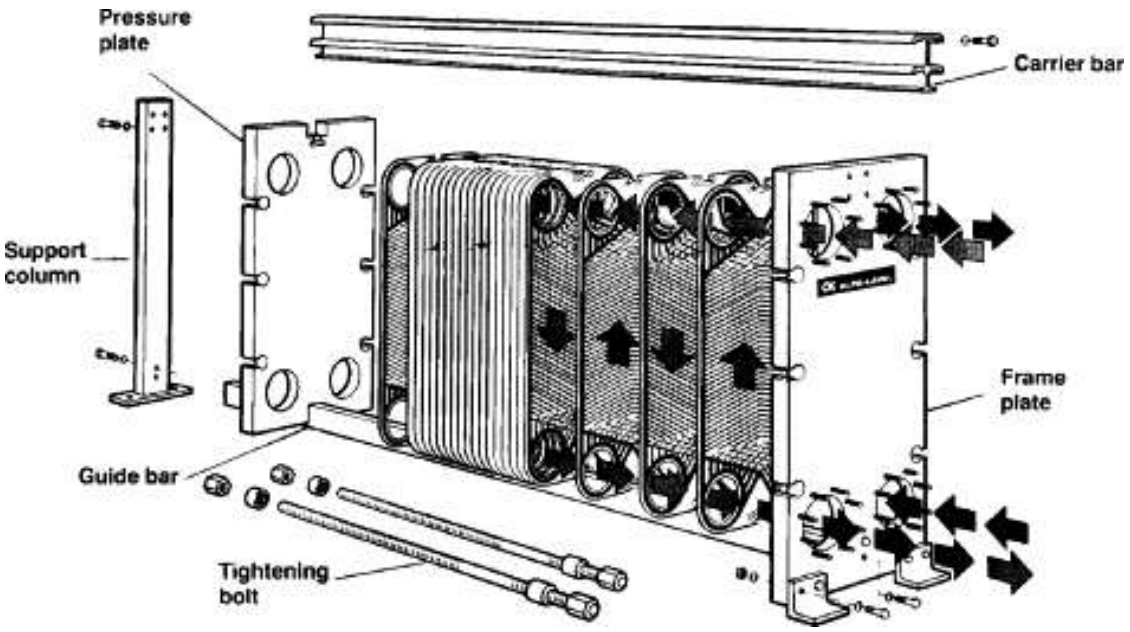


Fig: plate heat exchanger (Gösta Bylund, 2003)

The heating medium is hot water, and the cooling medium cold water, icewater or propyl glycol, depending on the required product outlet temperature. The plates are corrugated in a pattern designed for optimum heat transfer. The plate pack is compressed in the frame. Supporting points on the corrugations hold the plates apart, so that thin channels are formed between them. The liquids enter and leave the channels through holes in the corners of the plates. Varying patterns of open and blind holes route the liquids from one channel to the next. Gaskets round the edges of the plates and round the holes form the boundaries of the channels and prevent external leakage and internal mixing (Gösta Bylund, 2003).

2.2.4 Ultra pasteurisation

Ultra-pasteurisation can be utilised when a particular shelf life is required. For some manufacturers, two extra days are enough, whereas others aim for a further 30 – 40 days on top of the 2 – 16 days which is traditionally associated with pasteurised products. The fundamental principle is to reduce the main causes of reinfection of the product during processing and packaging, so as to extend the shelf life of the product. This requires extremely high levels of production hygiene and a distribution temperature. Lethal effect curves and time/ temperature curves for destruction of some enzymes and micro-organisms. 86 Dairy Processing Handbook/Chapter 6.1 of no more than 7 °C; the lower the temperature, the longer the shelf life. Heating milk to 125 – 138 °C for 2 – 4 seconds

and cooling it to < 7 °C is the basis of extended shelf life. ESL, Extended Shelf Life, is a general term for heat treated products which have been given improved keeping qualities by one means or another. Nevertheless, ESL products must still be kept refrigerated during distribution and in retail stores (Gösta Bylund, 2003).

2.2.5 UHT treatment

UHT is the abbreviation for Ultra High Temperature. UHT treatment is a technique for preserving liquid food products by exposing them to brief, intense heating, normally to temperatures in the range of 135 – 140 °C. This kills micro-organisms which would otherwise destroy the products. UHT treatment is a continuous process which takes place in a closed system that prevents the product from being contaminated by airborne micro-organisms. The product passes through heating and cooling stages in quick succession. Aseptic filling, to avoid reinfection of the product, is an integral part of the process. Two alternative methods of UHT treatment are used:

- Indirect heating and cooling in heat exchangers,
- Direct heating by steam injection or infusion of milk into steam and cooling by expansion under vacuum (Gösta Bylund, 2003).

2.2.6 Sterilisation in container

The original form of sterilisation, which is still in use, is in-container sterilisation, usually at 115 – 120 °C for some 20 – 30 minutes. After fat standardisation, homogenisation and heating to about 80 °C, the milk is packed in clean containers; usually glass or plastic bottles for milk, and cans for evaporated milk. The product, still hot, is transferred to autoclaves in batch production or to a hydrostatic tower in continuous production (Gösta Bylund, 2003).

2.3 Microbiology of milk

Milk, by its very nature, is a natural growth medium for microorganisms. Normally, milk is collected from a lactating animal (most commonly a dairy cow) at least twice a day and is recognized as a highly perishable foodstuff easily subjected to microbial contamination (Robinson). Microorganisms are always undesirable in milk and its products. These are capable of causing deterioration in flavour, physical appearance of milk and transmission of infectious diseases to the consumers. The various organisms get into milk through unhygienic, carelessness and unsanitary practices of the farmers, processors and distributors. Discoloration, sliminess, ropiness, putrefaction, rancidity and many other defects are caused by various microorganisms growing in the milk and milk products

(Maniruzzaman, Khan, Amin, Paul, & Islam, 2010). Bacterial contamination of raw and pasteurized milk is considered to be a great problem for dairy milk. The important genera of bacteria normally found in milk are, *Micro bacterium, Micrococcus, Streptococcus, Staphylococcus, Lactobacillus, Bacillus, Clostridium, Arthrobacter, Actinomyces, Coxiella, Pseudomonas* etc.(Adesiyun, 1994). Most of these organisms are free living, widely distributed in soil, feeds, cows, buffaloes, goats, dairy utensils etc. Contamination usually occurs at the farm where milk is produced (Maniruzzaman et al., 2010). Milk contains a wide range of nutrients including all of the vitamins, that can grow in milk may have very different properties. For some bacteria lactose is not a suitable energy source. Another relies on free amino acids as a nitrogen source, and fresh milk contains only tiny amounts of amino acids(P. P. Acharya, 2006). Consequently, such bacteria often start to grow after other bacteria have hydrolysed proteins, thus providing suitable nutrients. Another such example is the production of CO2 by some lactic streptococci, which stimulates growth of some lactobacilli. Milk contains natural inhibitors. Some bacteria do not grow in milk despite the presence of sufficient nutrients and suitable conditions. The important inhibitor found in milk is the immunoglobulins.(P. P. Acharya, 2006).

2.3.1 Thermoduric Bacteria

Milk is a nutritious medium that can support the growth of a large selection of bacterial contaminants. Bacteria are capable of utilizing the proteins, fats, carbohydrates and vitamins in milk for their growth and metabolism (Gleeson et al., 2013). Bacteria that contaminate milk include thermoduric bacteria that can survive pasteurisation and subsequently grow in the pasteurised milk or contaminate product (Gleeson et al., 2013). Thermoduric bacteria can survive exposure to pasteurization temperatures, and thermoduric psychrotrophic organisms can cause spoilage of pasteurize milk if stored at low temperatures(S.R. Tatini & Kaupp, 2002). The thermoduric bacteria commonly found on farm dairy equipment and in raw milk are limited to a few species of five groups of bacteria, viz., *streptococci, micrococci*, *coryneform bacteria*, aerobic spore formers and occasionally Gram-negative rods (W.H. Holzapfel & Cogan, 2020).

Elimination of thermodurics at milking is not feasible. Therefore, knowledge of their source and strategies for their reduction are important. The major sources of thermoduric in milk are contamination of the feat skin from soil and bedding, and subsequent contamination from deposits that can build up on milking equipment surfaces (Gleeson et al., 2013). Hygiene at milking can reduce the number of bacteria contaminating milk. Teat preparation at milking and a recommended plant cleaning procedure are critical to the prevention of the

contamination of milk with thermoduric bacteria (Gleeson et al., 2013).The shelf-life of milk products is defined as the period between manufacture or processing and when the consumer considers the product unsuitable for use. The product may be considered to be unsuitable for consumption, because of the presence of flavour defects and/or changes in physical appearance. The presence of thermoduric bacteria in raw milk are able to grow under refrigeration, e.g., *Bacillus cereus* and *B. circulans* are responsible in reducing the shelf life of the milk (J. Manners & Craven, 2003). The temperature of the product after heat treatment. Ideally, milk should be stored between 1 and 2 °C to minimize the growth of psychrotropic bacteria present in the milk from post pasteurization contamination. However, in many production situations, 1–2 °C is not practical, and the recommended temperature is 4–5 °C, with some countries permitting higher storage temperatures. Elevated temperatures allow bacteria to grow more quickly, thus reducing the time required for spoilage (J. Manners & Craven, 2003).

2.3.2 *Staphylococcus aureus*

S. aureus is a facultative anaerobic, Gram-positive coccus that is normally arranged in grape-like clusters. Cluster formation is due to successive cell division occurring in asymmetric three planes. The organism has a diameter of 1µm in average and liquid culture shows the arrangement of cocci in single, pairs, tetrads, or short chains of three or four cells. A few strains have capacity to produce capsules in young cultures (P. Chakraborty, 2005). They are non-motile and often golden-yellow pigmented cells. The primarily colonization sites are the anterior part of the nares and skin surfaces. The organism is non-spore forming but is resistant to dry conditions and high salt concentrations, which is essential when colonizing the skin surface. *S. aureus* is distinguished from the other species by its ability to clot blood plasma by the action of the enzyme coagulase (Ray, Morris, & Visick, 2012). *Staphylococci* can normally grow in basic media like nutrient agar and nutrient broth. However, in mannitol salt agar they form yellow colony by mannitol fermentation. They are non-motile and range in between 0.5 to 1.5µm in diameter (Shakya, 2019). *S. aureus* is one of the most important food-borne pathogens globally. It produces various toxins and invasive enzymes and can be found in numerous food products. Milk is an important source of staphylococcal food poisoning. After pasteurization, this microorganism or its enterotoxins might still remain in pasteurized milk. *S. aureu*s is a pathogen associated with serious community and hospital-acquired diseases. It has low nutritional requirements and widely exists in nature.

S. aureus produces a variety of toxins and invasive enzymes such as staphylococcal enterotoxins (SEs), hemolysins, Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), plasma coagulase, and deoxyribonuclease (Dai et al., 2019).

2.3.3 *Escherichia coli*

E. coli is a gram negative, rod shaped, facultative anaerobic and coliform bacterium belonging to the genus Escherichia which are commonly found in lower intestine of warm blooded animals (Tenaillon et al., 2012). They are non-sporulating bacteria which are about 2.0 µm long and 0.25-1.0 µm in diameter(Yu et al., 2014). Motile strains of *E. coli* have flagella which show peritrichous arrangement(Darnton, Turner, Rojevsky, & Berg, 2007). *E. coli* are grouped into enterotoxigenic, enteroinvasive, enteropathogenic and enterohaemorrhagic *E. coli.* At present around 190 strains of *E. coli* have been identified (Stenutz, Weintraub, & Widmalm, 2006). They produce gas as they can ferment some carbohydrates. They constitute about 0.1% of the gut microbiota along with other facultative anaerobes (Eckburg et al., 2005). *E. coli* is frequently contaminating organism, and is reliable indicator of fecal pollution generally in insanitary conditions of water, food, milk and other dairy products reported two cases of hemolytic uraemic syndrome which provide evidence that raw milk may be a vehicle of transmission of E. coli O157: H7, both affected persons consumed raw milk. Recovery of *E. coli* from food is an indicative of possible presence of enteropathogenic and/or toxigenic micro-organism which could constitute a public health hazard. Enteropathogenic *E. coli* (EEC) can cause severe diarrhoea and vomiting in infants and young children. *E. coli* O157:H7, which causes hemorrhagic colitis and hemolytic uremic syndrome, has been responsible for several outbreaks associated with consumption of unpasteurized and improperly processed pasteurized milk, and yogurt (Wang, Zhao, & Doyle, 1997).

2.4 Adulteration of milk

Milk is an important constituent of the human diet that is rich in proteins, carbohydrates, minerals, and vitamins, all of which are essential to human health. Due to its high nutritional value, global production and consumption of milk have increased, especially in the developing world (Food and Agriculture Organization of the United Nations, FAO). As a result, milk is a constant target of adulteration, causing not only economic loss, but also a significant risk to consumers' health (Carina F.Nascimento, Poliana M. Santos, Edenir Rodrigues Pereira-Filho, & Rocha, 2016). Possible reasons behind it may include-demand and supply gap, perishable nature of milk, and lack of suitable detection tests

(Azad & Ahmed, 2016). In addition, as an important feedstock for the food industry, cumulative effects can be observed in lacteous derivatives, thus increasing the demand for stringent evaluation of the product authenticity (Carina F.Nascimento et al., 2016).

Milk adulteration typically involves dilution or addition of inexpensive, low-quality, and sometimes dangerous products in order to increase the volume, mask inferior quality, or replace the natural substances in milk for economic gain. The simplest case is addition of water to increase the volume. Nowadays, more sophisticated frauds (and thus more difficult to detect) have been documented in both scientific and Gray literature, including addition of melamine to increase the nitrogen content of milk after dilution with water (Carina F.Nascimento et al., 2016). Other substances, such as formaldehyde, hydrogen peroxide, hypochlorite, dichromate, and salicylic acid have been added to increase the product shelf life, vegetable oils and surfactants are used for adulteration of the fat content as well as cheese whey and urea are used to artificially alter the protein content (Carina F.Nascimento et al., 2016).

Adulterants in milk mainly include addition of vegetable protein, milk from different species, addition of whey, table sugar and watering which are known as economically motivated adulteration. These adulterations do not pose any severe health risk (Azad & Ahmed, 2016). However, some adulterants are too harmful to be overlooked. Some of the major adulterants in milk having serious adverse health effect are urea, formalin, starch, detergents, ammonium sulphate, boric acid, caustic soda, benzoic acid, salicylic acid, hydrogen peroxide, sugars and melamine (Azad & Ahmed, 2016).

2.4.1. Milk Adulteration with Neutralizer

The addition of neutralizers like alkali bicarbonates, carbonates and hydroxides improves the shelf life of milk by neutralizing the developed acidity. Addition of neutralizers can cause increased mineral concentration in body fluids and soft organs leading to kidney stone development and commercial preparation of neutralizers might even be contaminated with heavy metals like arsenic, lead, etc. (Sowmya, Indumathi, Arora, Sharma, & Singh, 2015).

2.4.2 Milk Adulteration with Table Sugar

The common sugar present in milk is lactose. The fat content of the milk is more compared to the protein content. Table sugar like sucrose is added to the milk to increase the carbohydrate content of the milk and thus the density of milk will be increased. So, the milk can now be adulterated with water and it will not be detected during the

lactometer test. Ketose sugar will react with the resorcinol to give a red coloured precipitate, indicating the presence of Table sugar in milk (vlab.amrita.edu., 2011).

2.4.3 Milk Adulteration with Starch

Milk contains relatively large amount of fat. Addition of carbohydrate to milk increases its solid content. There by reducing the amount of fat present in the milk. Starch is one such component that is added to adulterate milk. The test to detect starch in milk uses iodine solution, addition of which turns the milk solution to blue black color due to the formation of starch –Iodo complex, in the presence of starch (vlab.amrita.edu., 2011).

2.4.4 Milk Adulteration with Formalin

Formalin is a preservative and can preserve milk for long period of time. Due to its high toxicity, it is considered to cause liver and kidney damage. Formalin reacts with Sulphuric acid and ferric chloride to give a purple-colored ring at the junction of the milk layers, thereby indicating the presence of formalin adulterated in milk (vlab.amrita.edu., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.2 Methodology

3.2.1 Study duration

The study was conducted from September 2019 to January 2020.

3.2.2 Laboratory set up

Laboratory setting was done in Microbiology laboratory, Central Campus of Technology, Dharan.

3.2.3 Area of study

Dharan Sub metropolitan city has 2112-hectare area and located in the eastern Terai of Nepal stretching from the edge of northern Mahabharat hill range up to the Charkoshe Jhadi in south separating from the southern Terai. The milk consumption by people of Dharan is high. The samples were collected from retail shops of different locations representing 20 wards of Dharan Sub metropolitan city.

3.2.4 Sampling method and sample size

Simple random sampling was done for the collection of samples and total of 20 pasteurized milk samples were collected from different places of Dharan.

3.2.5 Sample collection and Transport

Pasteurized milk (a packet of 5oo ml, refrigerated) samples marketed by 5 different dairy industries namely Kamdhenu, Gaubarsha, Dudhsagar, DDC and NMC dairy were purchased from different shops. All the samples were collected at morning (7-8), kept in ice box and transported to the laboratory. All samples were processed within 6 hours of receipt.

3.3 Chemical Analysis

Collected samples were tested for most common chemical adulterants (starch, neutralizer, table sugar and formalin) according to Manual of Methods of Analysis of Foods, Milk and Milk Products published by Ministry of Health and Family Welfare, Government of India, (FSSAI, 2016). Briefly, the tests were done as follows:

3.3.1. Starch test

Five ml of milk was boiled and then cooled and few drops of 1% iodine starch were added. The appearance of blue colour denoted positive test.

3.3.2 Neutralizer test

10 ml of sample to be tested and 10 ml of 95% alcohol was taken in test tube. Few drops of 0.1% alcoholic solution (w/v) rosalic acid was added and rossy red color indicate positive result.

3.3.3 Table sugar test

1 ml of milk and 1 ml of 0.5% resorcinol solution was mixed well and placed in boiling water for 3-5 minutes. The appearance of red colour denoted positive test.

3.3.4 Formalin test

Two ml of sample to be tested was taken and gently added equal volume of 90% H_2SO_4 containing traces of $FeCl_3$ from top of the test tube. Formation of purple ring at the junction indicated the presence of formalin.

3.4 Microbial analysis

For microbial analysis, collected samples were processed immediately after receipt. Briefly, tests were done as follows:

3.4.1 Total Viable Count (TVC)

TVC was performed according to Laboratory Handbook for Dairy Industry published by National Dairy Development Board (NDDB),2001 (NDDB, 2001). In which, serial ten-fold dilutions of the milk sample were done and TVC were determined by the pour plate method on nutrient agar and incubated at 37°C for 24 hours.

3.4.2 Total Coliform Count (TCC)

TCC was performed according to Laboratory Handbook for Dairy Industry published by National Dairy Development Board (NDDB), Nepal, 2001 (NDDB, 2001). Serial ten-fold dilutions of the milk sample were done and TCC were determined by the spread plate method on Mac-Conkey agar and incubated at 37°C for 24 hours.

3.4.3 Thermoduric bacterial count (TBC)

Thermoduric bacterial count was done following (Kimberly P Buehner, Sanjeev Anand, & Gemechis D Djira, 2015).

3.4.3.1 *E. coli*

One loopful each of the samples from 10-1 dilution was inoculated on to MacConkey Agar (MA). The plates were incubated at 37° C for 24 hours. Lactose fermenting colonies

on MacConkey agar were sub cultured to obtain pure culture. Pure cultures were tested biochemically (catalase test, oxidase test, Indole test, methyl red test, Voges Proskauer test, citrate utilization test, triple sugar iron agar test, urease test, oxidative fermentative test for confirmation of *E. coli* as described by (Isenberg, 2007) and (Cheesbrough, 2006).

3.4.3.2 *S. aureus*

Identification of *S. aureus* was done according to (S. P. Chakraborty, Mahapatra, & Roy, 2011). In which, one loopful of each sample was inoculated into Mannitol salt agar plates and incubated at 37ºC for 24 hours. Identification was done based on colony characteristics, Gram's staining, catalase test, oxidase test and coagulase test.

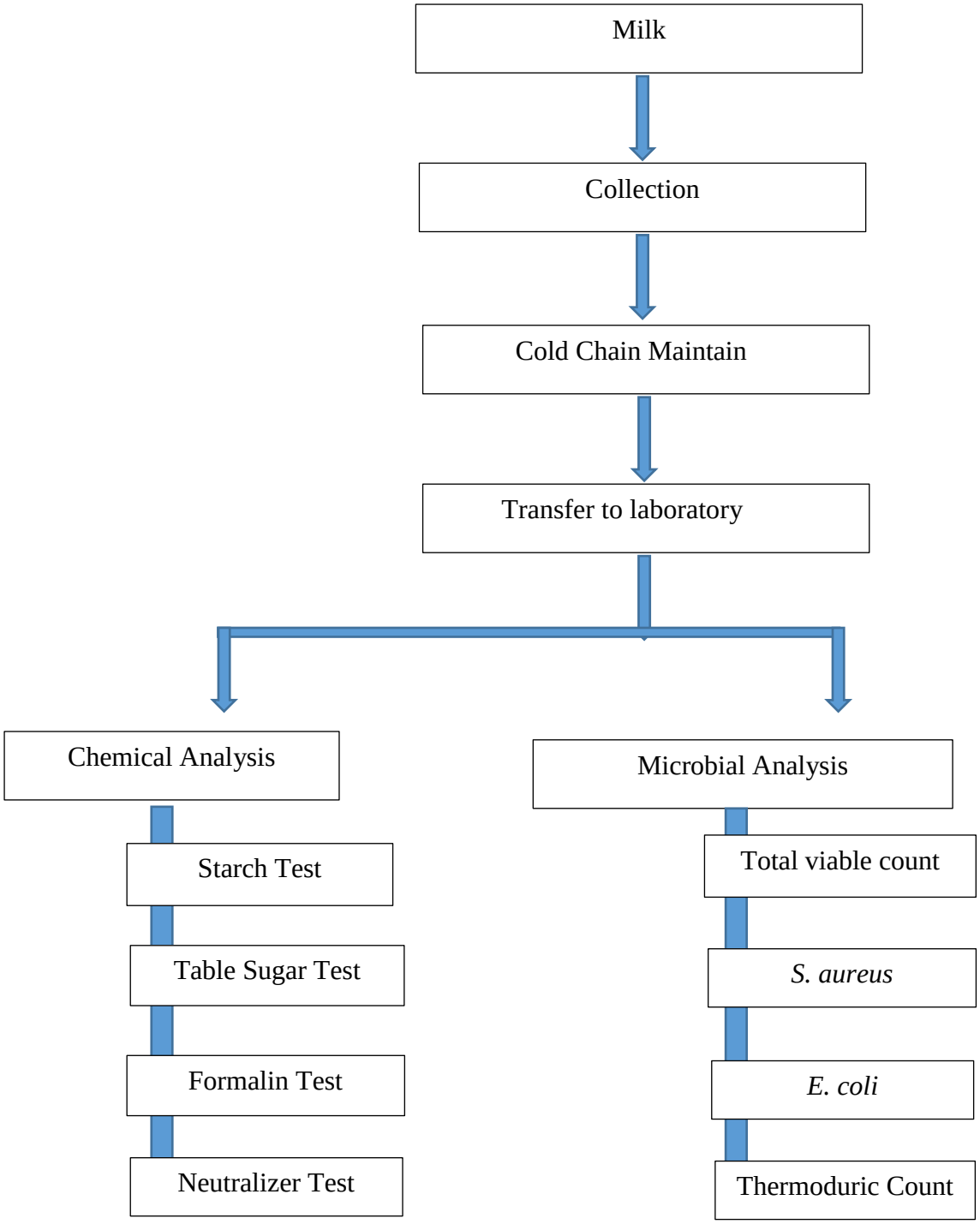


Fig 2: Flowchart of the experiment carried out.

3.5 Data Entry and Management

Obtained data were entered in Ms- Excel 2013 with proper coding system.

CHAPTER IV

RESULT

4.1 Microbial quality analysis of pasteurized milk

Milk samples from different dairy industries namely Dairy Development Corporation (DDC), NMC Dairy, Kamadhenu dairy, Gaubarsha Dairy and Dudhsagar dairy are collected at morning time, transported to the laboratory maintaining cold chain and analysed. Each analysis was performed at triplicates.

Results are tabulated as follows:

Here, P= Present, A=Absent, CFU= Colony Forming Unit

Table 3: Microbial quality of Dairy 1 milk

Sample/Date of collection	**Batch No.**	**Thermoduric bacteria (CFU/ml)**	***E. coli***	***S. aureus***	**Total Viable Count (CFU/ml)**	**Coliforms (Cells/ml)**
1 (2076/05/26)	No	Nil	P	P	11×10^4	16×10^3
2 (2076/06/02)	No	5×10^3	A	A	10×10^3	18×10^2
3 (2076/06/05)	No	35×10^2	A	A	5×10^3	11×10^2
4 (2076/06/06)	No	5×10^3	A	A	10×10^3	3×10^3
Nepal guideline		**-**	**Nil**	**-**	**-**	**Nil**
Remarks		Poor hygiene status, sanitation and pasteurization need to be implemented, should be well cooked before consumption.				

Table 4: Microbial quality of Dairy 2 milk

Sample/Date of collection	Batch No	Thermoduric bacteria (CFU/ml)	*E. coli*	*S. aureus*	Total Viable Count (CFU/ml)	Coliforms (Cells/ml)
1 (2076/06/06)	No	Nil	A	A	10×10^2	2×10^2
2 (2076/06/07)	No	Nil	A	A	22×10^2	2×10^2
3 (2076/06/08)	No	2×10^2	A	A	11×10^3	2×10^2
4 (2076/06/09)	No	18×10^2	A	A	10×10^2	21×10^2
Nepal guideline		**-**	**Nil**	-	**-**	**Nil**
Remarks		Poor hygiene status, sanitation and pasteurization need to be implemented, should be well cooked before consumption.				

Table 5: Microbial quality of Dairy 3 milk

Sample/Date of collection	Batch No.	Thermoduric bacteria (CFU/ml)	*E. coli*	*S. aureus*	Total Viable Count (CFU/ml)	Coliforms (Cells/ml)
1 (2076/06/01)	No	3×10^3	P	A	19×10^3	14×10^3
2 (2076/06/02)	No	2×10^3	A	A	18×10^3	74×10^3
3 (2076/06/05)	No	5×10^3	P	A	62×10^3	36×10^3
4 (2076/06/09)	No	5×10^3	A	P	13×10^3	TMTC
Nepal guideline		**-**	**Nil**	-	**-**	**Nil**
Remarks		Poor hygiene status, sanitation and pasteurization need to be implemented, should be well cooked before consumption.				

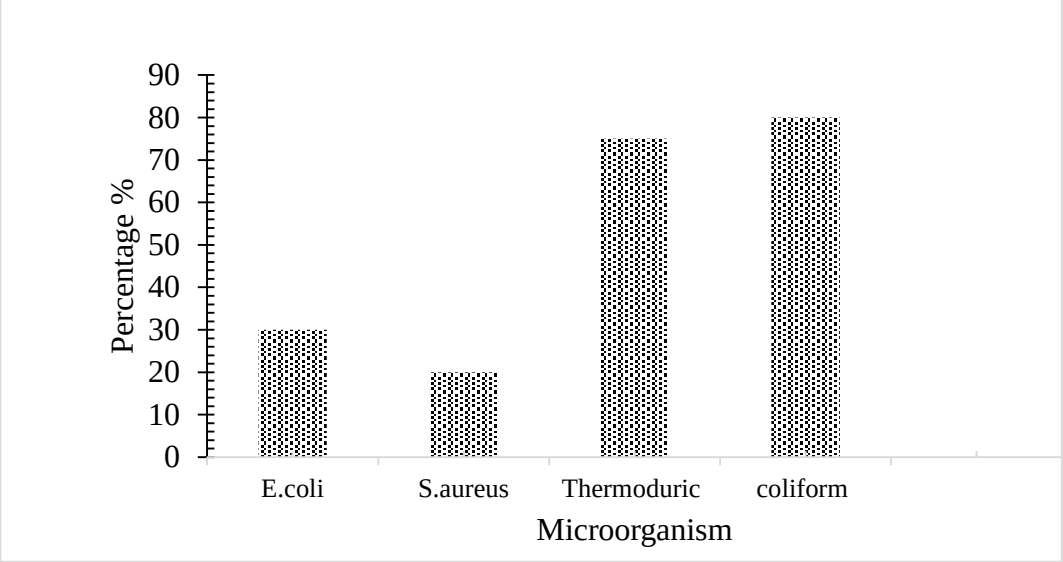
Table 6: Microbial quality of Dairy 4 milk

Sample/Date of collection	Batch No.	Thermoduric bacteria (CFU/ml)	*E. coli*	*S. aureus*	Total Viable Count (CFU/ml)	Coliforms (Cells/ml)
1 (2076/06/02)	No	Nil	A	A	TMTC	51×10^3
2 (2076/06/05)	No	2×10^3	A	A	19×10^3	16×10^3
3 (2076/06/06)	No	2×10^3	P	A	16×10^3	17×10^3
4 (2076/06/09)	No	6×10^2	P	A	17×10^3	17×10^3
Nepal guideline		**-**	**Nil**	**-**	**-**	**Nil**
Remarks	Poor hygiene status, sanitation and pasteurization need to be implemented, should be well cooked before consumption.					

Table 7: Microbial quality of Dairy 5 milk

Sample/Date of collection	Batch No.	Thermoduric bacteria (CFU/ml)	*E. coli*	*S. aureus*	Total Viable Count (CFU/ml)	Coliforms (Cells/ml)
1 (2076/05/27)	No	9×10^3	A	P	15×10^3	9×10^2
2 (2076/05/30)	No	10×10^3	P	A	17×10^3	11×10^3
3 (2076/06/01)	No	10×10^3	A	A	13×10^3	5×10^2
4 (2076/06/07)	No	8×10^3	A	A	14×10^3	32×10^3
Nepal guideline		**-**	**Nil**	**-**	**-**	**Nil**
Remarks	Poor hygiene status, sanitation and pasteurization need to be implemented, should be well cooked before consumption.					

4.1.1. Presence of Microorganisms shown in bar-diagram



4.2 Chemical analysis of pasteurized milk

Results are tabulated as follows:

Table 8: Chemical quality of Dairy 1 milk

Sample/Date of collection	Batch No.	Table sugar	Formalin	Starch	Neutralizer
1 (2076/06/06)	No	P	A	A	A
2 (2076/06/07)	No	P	A	A	A
3 (2076/06/08)	No	P	A	A	A
4 (2076/6/09)	No	P	A	A	A
Nepal guideline		**Nil**	**Nil**	**Nil**	**Nil**
Remarks		Table sugar added, absence of formalin, starch and neutralizer			

Table 9: Chemical quality of Dairy 2 milk

Sample/Date of collection	Batch No.	Table sugar	Formalin	Starch	Neutralizer
1 (2076/06/02	No	P	A	A	A
2 (2076/06/05)	No	P	A	A	A
3 (2076/06/06)	No	A	A	A	A
4 (2076/6/09)	No	A	A	A	A
Nepal guideline		**Nil**	**Nil**	**Nil**	**Nil**
Remarks		Table sugar added, absence of formalin, starch and neutralizer			

Table 10: Chemical quality of Dairy 3 milk

Sample/Date of collection	Batch No.	Table sugar	Formalin	Starch	Neutralizer
1 (2076/05/27)	No	P	A	A	A
2 (2076/05/30)	No	P	A	A	A
3 (2076/06/01)	No	P	A	A	A
4 (2076/06/07)	No	P	A	A	A
Nepal guideline		**Nil**	**Nil**	**Nil**	**Nil**
Remarks		Table sugar added, absence of formalin, starch and neutralizer			

Table 11: Chemical quality of Dairy 4 milk

Sample/Date of collection	Batch No.	Table sugar	Formalin	Starch	Neutralizer
1 (2076/06/01)	No	P	A	A	A
2 (2076/06/02)	No	P	A	A	A
3 (2076/06/05)	No	P	A	A	A
4 (2076/06/09)	No	P	A	A	A
Nepal guideline		**Nil**	**Nil**	**Nil**	**Nil**
Remarks		Table sugar added, absence of formalin, starch and neutralizer			

Table 12: Chemical quality of Dairy 5 milk

Sample/Date of collection	Batch No.	Table sugar	Formalin	Starch	Neutralizer
1 (2076/05/26)	No	P	A	A	A
2 (2076/06/02)	No	P	A	A	A
3 (2076/06/05)	No	P	A	A	A
4 (2076/06/06)	No	P	A	A	A
Nepal guideline		**Nil**	**Nil**	**Nil**	**Nil**
Remarks		Table sugar added, absence of formalin, starch and neutralizer			

4.2.1. Pie-chart showing presence of table sugar in pasteurized milk

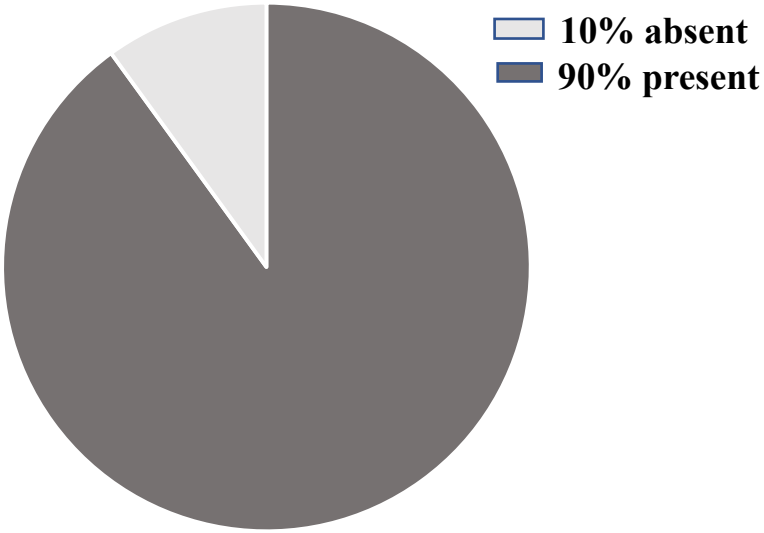
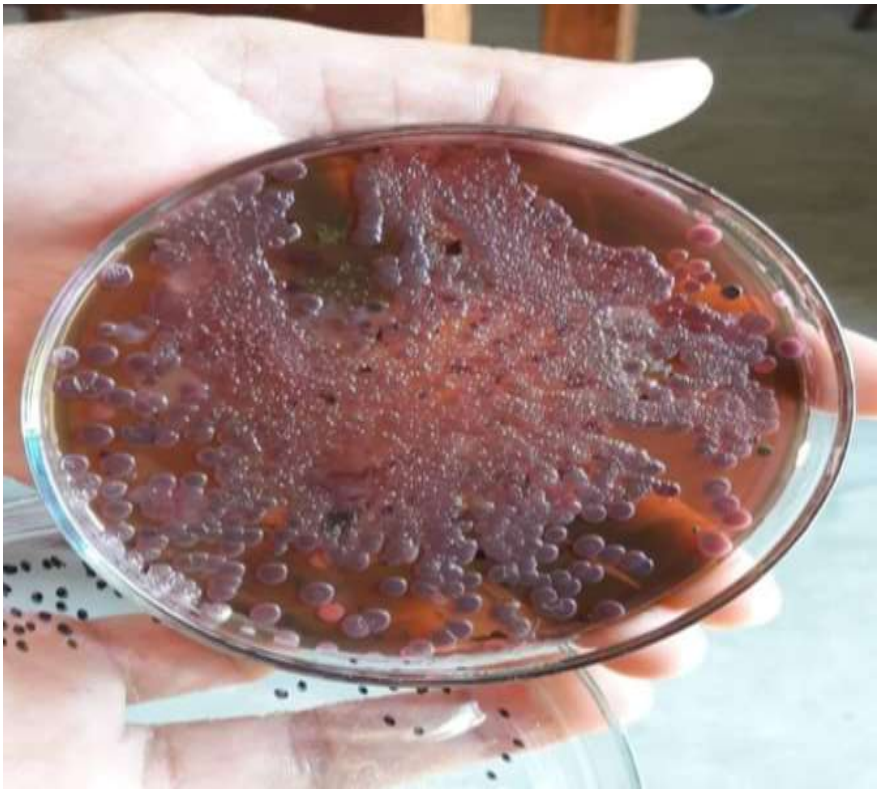


Fig: Pie-chart showing presence of table sugar in pasteurized milk

PHOTOGRAPHS

Photograph 1: Serial dilution of pasteurized milk sample



Photograph 2: Coliforms in Eosine Methylene Blue Agar

Photograph 3: *S. aureus* in Mannitol Salt Agar

Photograph 4: Thermoduric Bacteria in Plate Count Agar

CHAPTER V

DISCUSSION

The milk samples were tested for adulteration of starch, sugar, neutralizer, and formalin. Chemical analysis of the samples revealed that, most of the pasteurized milk were adulterated with table sugar. However, starch, formalin and neutralizer were absent in pasteurized milk samples. According to the Department of Food Technology and Quality Control (DFTQC) guideline for milk and milk products, adulterants should be absent in milk (DFTQC, 2011). However, this study found 90% of the pasteurized milk samples were adulterated with table sugar. In a similar study of (Parajuli, Rimal, Maharjan, Chaudhary, & Chaturwedi, 2018) reported that the extent of adulteration in milk of Kathmandu valley with table sugar and soda was 10% and 55% respectively. Findings of this shows that pasteurized milk marketed in Dharan are free from neutralizers. Since the neutralizers are added to neutralize the developed acidity (which in turn is due to increased microbial activity), it can be inferred that the quality of milk sold in Dharan is completely better than that of Kathmandu valley. Table sugar is commonly used as an adulterant to increase Solids-Not-Fat (SNF) level of milk. Starch was not found as an adulterant in the study by (Parajuli et al., 2018) and in this study too. The reasons behind not using starch as an adulterant could be the cost of starch. Among the pasteurized milk (total sample 20), E. coli, Total Coliforms, Thermoduric bacteria and *S. aureus* were detected in 30%, 80%, 75%, and 20% samples respectively. The average Total Viable Count (TVC) of pasteurized milk was 15×10^4CFU/ml. This result was greater than the findings of the previous study of (Al-Mazeedi Hani M., Gholoum Fadheelah A., & H., 2013) where, average counts of the aerobic bacteria in the pasteurized milk from three different dairy industries were 3×10^4 CFU/ml, 9×10^6 CFU/ml and 5×10^3 CFU/ml respectively. This finding also did not satisfy with international standard of European Union (EU, 2020) and Mandatory Nepalese Standard (MNS, 2016). Presence of higher load of bacteria in pasteurized milk may be due to inadequate pasteurization and post pasteurization contamination. It also indicated poor hygienic condition during packaging. In this study, none of the pasteurize samples were free from bacterial contamination. The average Total Viable Count (TVC) of pasteurized milk was 15×10^4 CFU/ml. The differences in findings of these studies can be correlated to difference in time as well as place. Higher bacterial counts indicate poor hygiene practice and ineffective

pasteurization of the milk (Harding, 1995). It suggests that proper handling of milk, improvement in sanitation, proper sterilization and disinfestations of contaminated utensils and use of safe water is mandatory for all stakeholders.

In this study, 80% of pasteurized milk samples were tested positive with an average Coliform count of 14×10^3 CFU/ml. This finding was higher than some previous studies (Silva et al., 2010); (El Nahas, Mohamed, El Barbary, & Mohamed, 2015) and (S. Acharya, Bimali, Shrestha, & Lekhak, 2017). The annual report published by DFTQC (2011/2012) reported that out of 65 milk and milk products analysed, 31 (47%) milk samples were found to be microbiologically unsafe (DFTQC, 2011). Hence, the result of this study complied with the study done by DFTQC showing that most of the milk being sold in Nepal might be microbiologically unsafe for consumption. The presence of coliforms in pasteurized milk sample may be due to defective pasteurization, adulteration of pasteurized milk with raw milk and unsanitary handling (Hasan, Islam, Mahmud, Uddin, & Ahmed, 2015). Similarly, current study found that the prevalence of *E. coli, S. aureus* and Thermoduric bacteria in pasteurized milk were 30%, 20%, and 75%. E. coli was reported in 18.75% and 20% of pasteurized milk samples by (S. Acharya et al., 2017) and (Parajuli et al., 2018) respectively which is just lesser than results of this study. S. aureus contamination was detected in 12.5%, 15% and 3.9% pasteurized milk (Arjyal, Dahal, & Khadka, 2004); (S. Acharya et al., 2017) and (Dai et al., 2019) and this result is a bit lesser than current study. The higher prevalence of S. aureus in pasteurized milk might be due to unhygienic processing, improper cleaning, deficient handling, and post-processing contamination of packaging material from the polluted environment (Sankhar, 2015). This study showed the average Thermoduric bacterial count of pasteurized milk was 4×10^3 CFU/ml. The average value of thermoduric bacteria in pasteurized milk reported by Delgado et al., (2013) was 3.19×10^2 CFU/ml. The variation on the thermoduric bacteria counts can be related to the difference in time and place. The presence of thermoduric bacteria in milk indicates that the pasteurizer may be defective so that some of the milk unable to reach the up to require pasteurizing temperature; there may be a high amount of foam which is not heated to temperature; or the vats may not be washed between runs (Rogers & Frazier, 1930). Different reports have suggested that pasteurized milk is contaminated with food-borne pathogens and it indicated that pasteurization alone could not be a stable solution to control the milk-borne diseases (Oliver, Jayarao, & Almeida, 2005). Hence, all the quality assurance systems such as Good Manufacturing Practice (GMP), Sanitation Standard Operating Procedure (SSOP),

and Hazard Analysis Critical Control Point (HACCP) should be implemented by industries. Our results demand further intense investigation and periodic monitoring of local milk vendors as well as dairy industries.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATION

6.1 Conclusions

Pasteurized milks sold in Dharan were found to be free from adulteration with starch, formaldehyde and neutralizers and thus present lesser chemical safety issue, for example, compared to milk sold in Kathmandu valley. The microbiological quality of pasteurized milk is still very poor. The presence of coliforms, thermoduric bacteria and *S. aureus* in pasteurized milk invites several speculations, ranging from faulty processing, to post-pasteurization contamination. Pasteurization is an effective technique to reduce and eliminate food-borne pathogens and other bacteria from milk. However, presence of food-borne pathogens in pasteurized milk indicates that pasteurization alone is not a certain solution for controlling milk-borne pathogens. To upgrade the quality of pasteurized milk, legal enforcement on the microbial guideline of marketed milk, routine monitoring of dairy industries and raw milk vendors, awareness campaign and good hygienic practice should be promoted.

6.2 Recommendations

The milk samples marketed in Dharan were found to contain high counts of microorganisms. The hygiene quality was unsatisfactory. The findings imply that people of these areas need to be careful about the quality.

Based on the above concluding remarks, the following Recommendations are forwarded.

1. Intensive training should be given to those personnel working in municipal, milking houses to ensure the hygienic practices during milking of animal.
2. Strict hygienic practices should be maintained to ensure contamination free milk while handling and processing.
3. Effective and adequate sanitation facility (wash basins, soap/ detergent, toilets, sanitized towels etc.) should be available on the milk shops and premises.

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APPENDICES

APPENDIX-I

LIST OF MATERIALS

A. Equipments Used

1. Autoclave
2. Weighting machine
3. Hot air oven
4. Incubator
5. Microscope
6. Refrigerator
7. Water bath shaker
8. Micropipette
9. Digital thermometer

B. Microbiological and biochemical media

1. Mannitol Salt Agar
2. Eosin methylene blue
3. Plate Count Agar
4. Nutrient Agar
5. Nutrient broth
6. Gelatin
7. MR-VP Broth
8. Simmons Citrate Agar
9. Triple Sugar Iron Agar
10. Sulphide Indole Motility Media
11. Urease agar
12. Peptone

C. Chemicals and Reagents

1. Catalase reagent (3% H_2O_2)
2. Crystal violet
3. Gram's Iodine
4. Alcohol
9. Alpha-napthol (5%)
10. Conc. Sulfuric acid
11. Safranine
12. Oxidase reagent

5. Ethanol
6. Kovacs reagent
7. Potassium hydroxide (40%) II
8. Rosalic acid
13. Lysol
14. Methyl red
15. Fecl3
16. Resorcinol Solution

D. Glasswares

1. Test tubes
2. Pipettes
3. Beakers
4. Petri plates
5. Conical flask
6. Glass rod and glass tubes
7. Reagent bottle
8. Slides
9. Measuring cylinder

E. Miscellaneous

1. Aluminium foil
2. Inoculating loop/ needles
3. Forceps
4. Cotton plugs
5. Cotton swab
6. Sample collecting bottles
7. Labelling tape
8. Measuring scale
9. Blotting paper
10. Test tube holder

APPENDIX-II

COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA.

1. Mannitol Salt Agar Ingredient	**Gm/litre**
Proteose peptone	10.00
Beef extract	1.00
Sodium chloride	75.00
D-mannitol	10.00
Phenol red	0.025
Agar	15.00
P H	7.4±0.2

Direction: 111.0 gm of the medium was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then, the medium was sterilized by autoclaving at 121°C (15 lbs. pressure) for 15 minutes.

2. Eosin Methylene Blue Ingredient	**Gm/litre**
Peptic digest of animal tissue	10.00
Dipotassium phosphate	2.00
Lactose	5.00
Sucrose	5.00
Eosin	0.40
Methylene blue	0.065
Agar	13.50
Final PH at 25°C	7.2±0.2

Direction: 36.96 gm of the medium was suspended in 1000 ml of distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121°C for 15 minutes.

3. Nutrient Agar Ingredient	**Gm/litre**
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.5
Yeast extract	1.5
Agar	15.00
Final P^H	7.2

Direction: 37 gm of the medium was suspended in 1000 ml of distilled water and boiled to dissolve completely. It was sterilized by autoclaving at 121°C for 15 minutes. For the preparation of Nutrient Broth Agar was not added.

4.Plate count Agar Ingredients	**Gm/liter**
Casein/tryptone	5.00
Yeast Extract	2.5
Glucose	1.00
Agar	15.00
P^H	7.20

Direction: 23.5 gm of the medium was suspended in 1000 ml of distilled water and boil to dissolve completely. Then sterilized by autoclaving at 121℃ for 15 minutes.

5.MacConkey Agar Ingredients	**Gm/litre**
Peptone	20.00
Sorbitol	10.00
Sodium chloride	5.00
Bile salt	1.50
Neutral red	0.03
Agar	15.00
Crystal violet	0.001
P^H	7.1±0.2

Direction: 51.531gm of medium was suspended in 1000 ml of distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121℃ for 15 minutes.

APPENDIX-III

Methods of Biochemical Test for the Identification of Bacteria

A. Catalase Test

This test is performed to detect the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme Catalase splits hydrogen peroxide to water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *streptococcus spp.*

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H2O2 was put on the surface of slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase or if an iron wire loop is used.

B. Oxidase Test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyses the transport of electrons between electrons donors. In the presence of redox dye Tetramethyl-p-phenylenediaminedihydrochloride, the cytochrome oxidizes it into a deep purple coloured end product Indophenol which is detected in the test. The test is used for screening *E. coli* which gives negative reaction.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent [what man's No. 1 filter paper impregnated with 1% tetramethyl-phenylenediaminedichloride]. The colony of the test organism was smeared on the filter paper. The negative test id indicated without the appearance of blue-purple colour.

C. Indole production Test

This test detects the ability of the organisms to produce an enzyme Tryptophanase. Tryptophan is oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia.

Procedure: The bacterial colony was inoculated on tryptone broth and them incubated at 37℃ for 24 hours, 1ml of Kovac's Reagent was added. Appearance of red ring at the top of the medium indicates positive indole test.

D. Methyl Red Test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red colour with the indicator methyl red and to overcome the buffering capacity of the system. Methyl red is an indicator which is already acid and will denote the change in degree of acidity by colour reactions over a ph range of 4.4-6.0

Procedure:

A pure colony of the test organism was inoculated into 2ml of MR VP medium and was incubated at 37℃ for 24 hours. After incubation about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red colour indicating acidity.

E. Voges-Proskauer Test

The principle of this test is to determine the ability of some microorganism to produce an acetyl methyl carbinol, a neutral end product or its reduction product 2,3- butanediol during fermentation of carbohydrates. An organism of Enterobacteriaceae group is either methyl red positive or Voges-Proskauer negative or methyl red negative and Voges-Proskauer positive.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37℃ for 24 hours. After incubation, about 5 drops of Barrett's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red colour.

F. Citrate Utilization Test

This test is performed to detect whether an organism utilizes citrate as the sole source of carbon. The utilization of citrate depends on the presence of an enzyme citrate produced by the organisms that breakdown the citrate to oxaloacetic acid and acetic acid which later

converted to pyruvic acid and carbon dioxide. Simmons Citrate Agar is used for this test, where sodium citrate is the only source of carbon and energy. Once the carbon dioxide is generated, it combines with sodium and water to form sodium carbonate an alkaline product, which changes the colour of the indicator (Bromomethyl Blue) from green to blue.

Procedure: A loopful of test organism was streaked on the slant area of the simmon's citrate agar medium and incubated at 37℃ for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. Bromothymol blue is green when acidic and blue when alkaline.

G. Urease Test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the colour of indicator incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37℃ for overnight. The positive organism shows pink red colour due to the breakdown of urea to ammonia. With the release of ammonia, the media becomes alkaline as shown by the change in colour of the indicator to pink.

H. Motility Test

This test id done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however, a few cocci forms are motile. Motile bacteria may contain single flagella. The motility media used for motility test is semi solid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in SIM medium and incubated at 37℃ for 48 hours. Motile organisms migrate from the stab line and diffuse into the medium causing turbidity. Whereas, non- motile bacteria show the growth along the stab line only.

I. Coagulase Test

Coagulase is an enzyme-like protein which causes clotting of human or rabbit plasma. The coagulase exists in two forms, free and bound coagulase. The extracellular free coagulase is a heat-labile enzyme. It requires the cooperation of plasma factor (coagulase reacting factor, CFR) for its clotting action. Bound coagulase is heat stable enzyme which reacts directly with the fibrinogen and causes aggregation of the staphylococci.

Procedure: The organism taken from Nutrient Agar plate was emulsified in a drop of water on a side and mixed with a drop of undiluted human plasma. Positive test is indicated by rapid clumping of the suspension. This test was done only for staphylococci.

J. Gelatine liquefaction test

This test is performed to detect microorganism that produces a proteolytic exoenzyme known as gelatinase, which causes hydrolysis [liquefaction] of gelatine. Gelatine is a protein which dissolves in warm water (50℃) and exists as a liquid above 25℃, and solidifies when cooled below 25℃. Once the degradation of gelatine occurs in the medium by an exoenzyme, it can be detected by observing liquefaction even at very low temperature (4℃).

Procedure: Using sterile inoculating loop the test organism was stabbed on the nutrient gelatine deep tubes from top to bottom. The inoculated tubes were incubated at 37℃ for 48 hours. After incubation, the tubes were placed into a refrigerator at 4℃ for 15 minutes. The tubes that remain liquefied show positive test for gelatine hydrolysis and those tubes that remain solid demonstrate negative rest.

APPENDIX -IV

Table A1: Biochemical characterization of *S. aureus*

Biochemical test	Reaction
Catalase	+
Coagulase	+
Indole	-
Methyl red	+
Voges-Proskauer	±
Citrate utilization	-
Gelatine liquefaction	+

Table A2: Biochemical characterization of *E. coli*

Biochemical test	Reaction
Catalase	+
Indole	+
Methyl red	+
Voges-Proskauer	-
Citrate utilization	-
Gelatine liquefaction	-
SIM	+
Urease activity	-