CHAPTER –I

1. INTRODUCTION

1.1 *DAHI* OR YOGHURT

Turkish yoghurt, Egyptian laban, Arabian leben, far eastern saya, Armenian matzoon and Indian Dahi is similar fermented milk products made from pasteurized or boiled milk by inoculation of harmless lactic acid bacteria (Flora, 1996; Sukumar, 2002).

Dahi is defined as the milk product by using the pasteurized or boiled milk of cow or buffalo by inoculating the starter of lactobacilli to milk of lukewarm temperature so that acidification and curdling occur naturally by the used harmless lactic acid bacteria. If it is prepared from the skimmed milk, solid not fat (SNF) should retain from the milk it is prepared. If not mentioned the milk fat or SNF should be equal to the milk of buffalo. *Dahi* may contain the added cane sugar or edible spices. In cane sugar or spices added *Dahi* the SNF should be retained to the milk from which it is prepared excluding the added cane sugar or spices (DFTQC, 2003).

Yoghurt is the fermented milk product by the action of symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in pasteurized milk resulting in the reduction of p^H to not above 1% lactic acid with or without coagulation (iso-electric precipitation) (Codex, 2003).

All foods have the potential to cause food borne illness; milk and milk products are no exception. Dairy animals may carry human pathogens. Such pathogens present in milk may increase the risk of causing food borne illness. Moreover, the milking procedure, subsequent pooling and the storage of milk carry the risks of further contamination from man or the environment or growth of inherent pathogens. Potential also exists for the contamination of milk with residues of veterinary drugs, pesticides and other chemical contaminants. In many countries milk and milk products form a large portion of the diet

of consumers especially infants, children, and pregnant and lactating women (FAO, 2004).

“Illness due to contaminated food was perhaps the most widespread health problem in the contemporary world, and “an important cause of reduced economic productivity” (WHO, 2000). Hundreds of millions of people suffer from communicable diseases caused by contaminated food and drinking water. “Access to nutritionally adequate and safe food is a right of each individual.” Food was a major vehicle for the transmission of environmental contaminants both chemical and biological to human populations throughout the world (WHO, 2000). Approximately 1.5 billion episodes of diarrhea occur annually in children under the age of 5, resulting in some 1.8 million deaths. It is estimated that up to 70 percent of diarrheal episodes may be caused by food-borne contaminants. Although many different pathogens have been identified, food contaminated with pathogenic *E. coli* causes up to 25 percent of all diarrheal episodes in infants and children, while *Campylobacter jejuni* and *Shigella* spp. account for 10-15 percent and 5-15 percent respectively (WHO, 2000).

Common milk borne diseases are listeriasis, *Bacillus cereus, Staphylococcus aureus* intoxications, brucellosis, tuberculosis, typhoid, cholera, campylobacteriosis, *E. coli* gastroenteritis, salmonellosis, shigellosis, typhoid and paratyphoid fevers, amoebiasis and other gastrointestinal tract disorders (Käferstein, 2003)

The nutritive values and delicious taste of this traditional dairy product has made favorite to the people of all ages. However, most of consumers are found to be unaware of the microbiological quality of that product (Udas, 1996). The microbiological quality of food refers to the safe level of microbiological standards which should be as high as possible (Robinson, 1983). Microbiological criteria represent one form of acceptance criteria. The establishment of meaningful and logic microbiological criteria is a complex process. Microbiological criteria define the acceptability of a product based on the presence/absence or number of microorganisms (and/or their toxins) per unit(s) of mass, volume, area or lot. (ICMSF, 1997)

A number of food borne outbreaks have been reported by consumption of inferior quality of dairy products all over the world (Yadav, 1993). Among the leading diseases of Nepal food borne diseases are the most common in which gastroenteritis, diarrhea, food poisoning, typhoid fever and dysentery are directly related to the consumption of food (DoHS, 2005/2006) in which the consumption of most common dairy product i.e. *Dahi* can't be neglected.

The quality of milk and milk products, particularly in Nepal, depends very much on the seasonal and as well as spatial variations. In the context of Nepal the microbiological quality of dairy products was very poor in terms of TPC, coliform count and yeasts and moulds count. Poor quality of raw milk, poor technology of processing, lack of cooling facilities at production level retail and marketing level as well as post pasteurization contaminations, are the main cause of poor quality (Waldhauer, 2001).

In developing countries like Nepal, neither there is strict food act nor do proper food inspection and regular monitoring program to complain or judge with the set of national and international standard. Hence there is very much likely to use such milk which may have been contaminated at any step of manufacturing and marketing. Besides in Nepal curd and yoghurt are made in cottage industries like restaurants, comfit shops and in small dairy farm in traditional ways and in the customary they have learned from their family and guide. These traditional curd manufacturer and handlers are very less aware in sanitation, handling and personal hygiene (Udas, 1996). Further more, neither these companies are applying GMP, GHP, and ISO, neither HACCP nor the Nepal standard. These all facts support the microbial contamination of milk and milk products with undesirable microorganisms. Yoghurt or curd are readymade foods and are consumed directly without pretreatment like heating, washing, etc. Therefore, the consumption of such contaminated dairy products could directly affect the consumers (Wealthier, 2001).

In Nepal very few research have been done in quality of *Dahi*, For quality measurement nutritional value and labeling are more prioritized and microbiological quality and thus

people safety has been neglected. No comprehensive microbial national standards for *Dahi* are available.

Therefore, this study is designed to evaluate the microbial quality of *Dahi* of Kathmandu valley in terms of coliform count, *Bacillus cereus* count, Staphylococci count, *Salmonella*, *Shigella* detection and yeast and mould count. These counts assess the microbiological, safety and quality of *Dahi*.

This study hence will give the microbiological status of *Dahi* of Kathmandu. Besides, it will examine the microbiological quality of *Dahi*, it could recommend the authorized agency to give concern to improve the standards of *Dahi*.

CHAPTER –II

2. OBJECTIVES

2.1. GENERAL OBJECTIVE

To evaluate the Microbiological quality of *Dahi* of Kathmandu on the basis of detection and counting of Starter, indicator organisms, spoilage organisms and pathogens.

2.2. SPECIFIC OBJECTIVES

- To study the storage and selling practice of *Dahi*.
- To isolate, enumerate, and identify the principal starter *Streptococcus thermophilus* and to observe its urease activity.
- To enumerate and identify the indicator organisms such as coliforms (fecal contamination), *Geotrichum candidum* (Dairy plant machinery sanitation) and Staphylococci (indicator of handling).
- To enumerate, isolate and identify food borne pathogens such as *Salmonella* spp., *Shigella* spp. and *Bacillus cereus.*
- To perform the yeast and moulds count and to identify of isolated moulds.

CHAPTER –III

3. LITERATURE REVIEW

3.1. DEFINITION OF *DAHI* / YOGHURT

According to the PFA rules (2006), *Dahi or curd* are the products obtained from pasteurized or boiled milk by souring natural or otherwise, by harmless lactic acid or other bacterial cultures. *Dahi* may contain added cane sugar. *Dahi* should have the same minimum % of milk fat or milk solid not fat as the milk from which it is prepared. Where *Dahi* or curd, other than skimmed milk *Dahi*, is sold or offered for sale without any indication of the class of milk the standards prescribed for *Dahi* prepared from buffalo milk shall apply.

The microorganisms in the final products must be viable and abundant. It may contain skimmed milk powder, milk powder, unfermented butter milk, concentrated whey, whey powder, whey protein, whey protein concentrate, water soluble milk proteins, edible casein and caseinates manufacture from pasteurized product. It may also contain sugar, corn syrup or glucose syrup in the sweetened yoghurt and fruits in fruits yoghurt. It shall have smooth surface thick consistency without separation of whey. It should be free from vegetable oil/fat, animal body fat, mineral oil and any other substance foreign to milk (PFA, 2006).

3.2 HISTORY OF *DAHI* / YOGHURT

Nobody knows when Yogurt was discovered, its origins have been lost in the mists of time. What we do know for sure is that fermented milk was already being used in prehistoric times or about the Neolithic Period (approximately 10,000 B.C.), Yoghurt is one of the oldest foods known to man and has been a basic nutritional product in south-eastern Europe, the Middle East, Central Asia and parts of the Far East for thousands of years. It is unquestionable that yoghurt existed many years before man wrote about it. It is likely that its discovery was accidental. It is believed that it first appeared in the

Middle East, in an area now part of Turkey or possibly in neighboring Persia. The warm climate of the Middle East, in combination with the lack of hygienic conditions offered an ideal environment for the yoghurt bacillus to appear and to multiply naturally. It is believed that yoghurt subsequently spread from the Middle East to more distant regions with the development of trade and the waging of wars.

The import of yoghurt to Western Europe is said to have occurred in the 16th century. An Armenian doctor cured the French king Francis I, who suffered from melancholy and intestinal problems with this unique food, whilst others state that a Jewish healer from Constantinople arrived on foot with a flock of sheep and goats. It seems that this fact became the reason why the French named yoghurt "the milk of eternal life". Yoghurt was little known in Western Europe and America even up to the 1920's and 1930's (Flora, 2004).

The nutritional as well as health promoting properties of yoghurt were only known after the study done by prominent French bacteriologist of Russian origin, Ilya Ilyich Metchnikov, a researcher at the Pasteur Institute in Paris in 1900 AD who was awarded the Nobel Prize in 1908. He isolated the starters such as lactobacilli and their beneficial role in gastro intestine. He found that long life span of Bulgarian tribe in comparison to the people of other part of world was due to consumption of yoghurt which the colon of intestine healthy and free of pathogens (Flora, 2004).

In context of Indian sub-continent, existence of curd or *Dahi* is described in the *Vedas* and in the *epics* as Makhan chori by lord Krishna in Dwaper era (Sukumar, 2002). In Nepal the Sovereigns pioneer Gopalbansi and Mahispalbansi used to keep cows and buffalos for production of milk, curd (*Dahi*), and ghee. They offered these products to gods (Upadhyay *et al*., 2001).

3.3 STARTERS OF *DAHI* / YOGHURT

Harmless lactic acid bacteria are the Starters of yoghurt and yoghurt like fermented milk products**.** Symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* are the starters of yoghurt. Yoghurt like fermented milk

products like acidophilus milk, kefir, kumys use starters of *Lactobacillus acidophilus,* any *Lactobacillus* species, *Bifidobacterium bifidus*, *Leuconostoc* spp. and *Acetobacter* spp. *(codex stan- 2003) Streptococcus lactic, S. diacetilactis* and *S. cremoris* single or in combination with *Leuconostoc* spp. can be used as starter of Sweet *Dahi*. Sour *Dahi* contain *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in addition of the starters of Sweet *Dahi* (PFA, 1995). *Dahi* contain harmless *Lactobcillus* spp. as starter culture (DFTQC, 2006).

Starter cultures are those microorganisms that are used in the production of cultured dairy products such as yogurt and cheese. The natural microflora of the milk is inefficient, uncontrollable, and unpredictable, or is destroyed altogether by the heat treatments given to the milk. A starter culture can provide particular characteristics in a more controlled and predictable fermentation. The primary function of lactic starters is the production of lactic acid from lactose. Other functions of starter cultures may include the following:

- flavour, aroma
- proteolytic and lipolytic activities
- inhibition of undesirable organisms (Robinson, 1983)

There are two groups of lactic starter cultures: 1. simple or defined: single strain, or more than one in which the number is known; **2.** mixed or compound: more than one strain each providing its own specific characteristics (Robinson *et al*., 1986).

Starter cultures may be categorized as mesophilic or thermophilic:

Mesophilic

- *Lactococcus lactis* subsp. *cremoris*
- *L. delbrueckii* subsp. *lactis*
- *L. lactis* subsp. *lactis* biovar *diacetylactis*
- *Leuconostoc mesenteroides* subsp. *cremoris*

Thermophilic

- *Streptococcus salivarius* subsp. *thermophilus* (*S.thermophilus*)

- *Lactobacillus delbrueckii* subsp. *bulgaricus*
- *L. delbrueckii* subsp. *lactis*
- *L. casei*
- *L. helveticus*
- *L. plantarum* (Robinson *et al*., 1986).

3.4 COMPOSITION OF *DAHI* / YOGHURT

Yoghurt should have minimum of 2.7 % (m/m) milk proteins, less than 15%(m/m) milk fat, minimum of 0.6 % titrable acidity, expressed as % lactic acid (%m/m), sum of microorganisms constituting the starter cultures minimum 10^7(cfu/g in total), yeasts 0cfu / ml, minimum of 50% of dairy ingredients (Codex, 2003).

Table no.1 Composition of whole milk *Dahi* (percentage)

Water	Fat	Protein	Lactose	Ash	Lactic acid	Total solid
85-88	5-8	3.2-3.4	4.6-5.2	0.70-0.72	0.5-1.1	12-15

(Sukumar**,** 2002)

Dahi should contain the same percent of fat and SNF from the milk it is prepared. If it is prepared from skimmed milk it should be specified and most satisfy provision of Fat and SNF for skimmed milk (DFTQC, 2006).

3.5 MARKET QUALITY OF *DAHI*

Qualities **Requirements**

Color: Yellowish creamy-white for cow and creamy white for buffalo milk; free from browning.

Appearance: Smooth and glossy surface, creamy layer on top with whole milk product free from extraneous matter.

Flavor: Mild, pleasant smell, clean acid taste free, from off flavor,

Body: Soft and firm, free from gas-holes and whey pockets

Acidity: 0.75 to 0.85 % of lactic acid (Sukumar, 2002).

3.6 PERMITTED INGREDIENTS

The minimum dairy ingredients content requirement of 51 percent of the total weighs of yogurt. Sodium chloride; and Non-dairy ingredients (such as nutritive and non nutritive sweeteners, fruits and vegetables as well as juices, purees, pulps, preparations and preserves derived there from, cereals, honey, chocolate, nuts, coffee, spices and other harmless natural flavoring foods) Gelatin and starch in: fermented milks heat-treated after fermentation; flavored fermented milk; and - plain fermented milks if permitted by national legislation in the country of sale to the final consumer (Codex, 2003).

They are added only in amounts functionally necessary as governed by Good Manufacturing Practice, taking into account any use of the stabilizers/thickeners. These substances may be added either before or after adding the non-dairy ingredients (Codex, 2003**).**

It may contain skimmed milk powder, milk powder, unfermented butter milk, concentrated whey, whey powder, whey protein, whey protein concentrate, water soluble milk proteins, edible casein and caseinates manufacture from pasteurized product. It may also contain sugar, corn syrup or glucose syrup in the sweetened yoghurt and fruits in fruits yoghurt. It shall have smooth surface thick consistency without separation of whey. It should be free from vegetable oil/fat, animal body fat, mineral oil and any other substance foreign to milk (PFA, 2006). *Dahi* may contain added cane sugar or spices (DFTQC, 2006).

3.7 TYPES OF *DAHI* / YOGHURT

3.7.1On the basis of fat content

On the basis of fat content yoghurt can be divided in to whole milk yoghurt, low fat yoghurt and non fat yoghurt. Whole milk yoghurt contains fat equal to the milk from which it is prepared, low fat yoghurt with less than 3 grams of fat and non fat yoghurt with less than 0.5 grams per reference amount (FDA, 1996).

3.7.2 On the basis of taste

Table no. 2 type of yoghurt on the basis of taste

Desig-nation	Starters	Acidity lactic acid (%wt.) (max)	Yeast and Mould count Per gram (max.)	Coliform cfu / ml (max.)	Phosphatase test
Sweet *Dahi*	*S.lactis, S.diacetilactis S. cremoris* (single or in combination with or with out *Leuconostoc* spp.)	0.70	100	10	-ve
Sour *Dahi*	Same as above, along with *L. bulgaricus* or *S. thermophilus* or both	1.0	100	10	-ve

(Sukumar**,** 2002)

3.7.3 On the basis of purpose of manufacture:

A. For churning into desi (or indogenious) butter (makkhan); it is mainly made from whole milk.

B. For direct consumption (Sukumar**,** 2002). *Dahi* can be prepared from skim milk or from whole milk so the fat content of *Dahi* is being the basis for type of *Dahi* (DFTQC, 2006).

3.7.4 On the basis of set and texture: Set yogurt and stirred yogurt

Set yogurt: A solid set where the yogurt firms in a container and not disturbed.

Stirred yogurt: Yogurt made in a large container then spooned or otherwise dispensed into secondary serving containers. The consistency of the "set" is broken and the texture is less firm than set yogurt. This is the most popular form of commercial yogurt (Frye, 2004)

3.7.5 On the basis of flavoring ingredients: Plain yoghurt, flavored yoghurt

Plain yogurt is made from whole milk, low fat or nonfat milk without additional flavoring ingredients. Flavored yogurt has sugar and either artificial flavorings or

natural fruit (or both) added. Some flavored yogurts contain gelatin or stabilizers for a thicker texture. Fruit-flavored yogurts can either have the fruit on the bottom (to be mixed in by the consumer) or be already stirred-in which case they're referred to as *Swiss-style*. Frozen yogurt-which resembles soft-serve ice cream in texture-has become very popular and competes head-to-head in some markets with ice cream (Robinson *et al*., 1986).

3.7.6 On the basis of heat treatment: Live active yoghurt and heat treated yoghurt. Live active yoghurt contains starters of 10^7 cfu / ml to the date of minimum durability. Heat treated yoghurt contains no live starters. It is generally done to increase its shelf life (FDA, 1996).

Table 3 Type of yoghurt on the basis of milk used

VARIOUS TYPES OF YOGHURT				
Contents	Yoghurt prepared from cow milk			Yoghurt with fruit (%)
	Whole (%)	Partially skimmed milk (%)	Skimmed milk (%)	
Water	87	89	8	81
Proteins	3.5	3.4	3.3	2.8
Lipids	3.9	1.7	0.9	3.3
Glucides	3.6	3.8	4	12.6
Lactic acid	1.2	1.2	1.2	1.2
Ashes	0.7	0.72	0.75	0.7
Alimentary	0	0	0	-
Energetic	63	43	36 kcal	88kcal

(Flora, 2004)

3.8 PROVISIONS FOR YOGHURT

- A single standard of identity for yogurt, which includes provisions for low fat and nonfat yogurts.
- A minimum of 10^7 colony forming units per gram (CFU/g) of live and active characterizing cultures at the time of manufacture of yogurt.
- An acidity of pH 4.6 or lower, rather than the requirement of titrable acidity expressed as lactic acid in yogurt.
- The use of optional milk-derived ingredients after pasteurization and culturing of yogurt.
- The use of reconstituted dairy ingredients and WPC (whey protein concentration) as basic dairy ingredients in yogurt, and the specifications related to WPC, when used.
- The optional use of any milk derived ingredient that provides a technical or functional purpose in yogurt.
- The minimum dairy ingredients content requirement of 51 percent of the total weight of yogurt.
- The use of any safe and suitable nutritive or non-nutritive innovative sweeteners in yogurt.
- The use of safe and suitable emulsifiers in yogurt.
- The use of safe and suitable preservatives in yogurt.
- The use of any safe and suitable ingredient added for a nutritional or functional purpose in yogurt (Frye, 2004).

3.9 BIOCHEMISTRY OF YOGHURT FORMATION

Yoghurt is the famous dairy product which is obtained by biological spontaneous or controlled fermentative acidification of milk by *Lactobacillus delberki subsp bulgaricus* and *Streptococcus thermophilus* in combination with or without other harmless lactic bacteria or yeasts. These starters possess enzyme β - galactosidase which hydrolyses lactose the major sugar of milk in to glucose and galactose. These organisms further ferment the sugar into lactic acid. These lactic acids develop acidic condition. Acidic

condition causes denaturation of milk proteins (casein). Denatured proteins fold to form curd. These cultures are alive and abundant two millions per gram in quality yoghurt. The lipidic part of this fermented product remains almost identical to that of original milk. These starters partially hydrolyses milk proteins therefore, more digestible. Lactic acid solubilizes the phosphate and calcium salts of milk and are immediately available and easily assimilated. Final refrigeration debilities the fermentative capacity of starters and can be preserved without spoilage (Flora, 2004).

The following overarching principles apply to the production, processing and handling of all milk and milk products:

From raw material production to the point of consumption, dairy products produced should be subject to a combination of control measures, and these control measures should be shown to achieve the appropriate level of public health protection. Good hygienic practices should be applied throughout the food chain so that milk and milk products are safe and suitable for their intended use.

Wherever appropriate, hygienic practices for milk and milk products should be implemented within the context of HACCP. In the case where HACCP cannot be implemented at the farm level, good hygienic practices, good agricultural practices and good veterinary practices should be followed. Control measures should be validated as effective**.** The overall effectiveness of the system of control measures should be subject to validation. Control measures or combinations thereof should be validated according to the prevalence of hazards in the milk used, taking into consideration the characteristics of the individual hazards(s) of concern and established Food Safety Objectives and/or related objectives and criteria (Codex, 2003).

3.10 FACTORS RESPONSIBLE FOR SUBSTANDARD MICROBIOLOGICAL QUALITY OF *DAHI* are:-

a. Use of low quality of raw materials (raw milk, and ingredients added to curd).

b. Use of substandard starter cultures.

c. Unfavorable temperature of incubation.

d. Contamination from badly cleaned utensils.

e. Post manufactures contamination

f. long and bad storage practices (Robinson *et al*., 1986).

3.11 USES OF *DAHI*

A) Whole milk *Dahi*

i) For direct consumption either as such with salt/sugar, or as beverage after beating the curd and mixing with water, salt sugar, fruits etc. called lasi

ii) For preparation of chakka and srikhanda.

iii) For preparation of makkhan

B) Skim milk *Dahi*: For the direct consumption; especially by heart patients since it is low in fat and by the low-income group of the population because it is cheap yet nutritious (Sukumar, 2002).

C) *Dahi* is consumed for treatment of some diseases like diarrhea, dysenteries as medicine. In hindu religion *Dahi* is offered to gods which in turn bring health, happiness and prosperity for the householders (Upadhyay *et al*., 2001).

3.12 PRESERVATION OF YOGHURT

Different methods are used for preservation, refrigeration, freezing, pasteurization, carboration etc. Refrigeration, if conventionally prepared *Dahi* is allowed to stand at room temperature or prolonged storage it becomes highly acidic due to continuous acidification by starters or contaminates. This is accomplished by whey formation, making the product unfit for human consumption. Under refrigeration temperature (5-10^0C), it usually keeps well for a maximum period of one week (Sukumar, 2002).

Freezinng is used for preservation of yoghurt and thus preserved yoghurt is called frozen yoghurt. The specific Starter cultuers can be raeactived in the responsible number by thawing. The frozen product is named as such and is sold for direct consumption, only (codex 2003).

Dahi or yoghurt can be preserved by pasteurization or heat treatment after fermentation and is called pasteurized or heat treated yoghurt. Such yoghurt has extended shelf life even at room temperature (Robinson, 1983).

Carbonated *Dahi* is made by bubbling of carbon dioxide to starters inoculated milk bottles at 1 psi for one minute and are crown- crocked. These bottles are incubated for 16 to 18 hrs at 25 to 30^0C until firm curd is obtained. Carbonated yoghurt has self life of 15 to 30 days without refrigeration (Sukumar 2002).

3.13 PACKAGING AND STORAGE OF *DAHI* / YOGHURT

The traditional container for *Dahi* is an earthenware cups. However, modern packaging includes glass bottles, plastic cups and bottles and plastic bages. The recommended storage temperature is around 5 to 10^0C (Sukumar, 2002).

3.14 ESSENTIAL COMPOSITION AND QUALITY FACTORS

Raw Materials

Milk and/or products obtained from milk, Potable water for the use in reconstitution or recombination (Code, 2003).

3.15 BENEFITS OF *DAHI*

In many modern societies, fermented dairy products make up a substantial proportion of the total daily food consumption. Furthermore, it has long been believed that consuming yogurt and other fermented milk products provides various health benefits. Studies from the 1990s on the possible health properties of yogurt added to this belief. Yogurt is one of the best-known of the foods that contain probiotics (Perdigon *et al*., 1995).

The benefits of yogurt and Lactic acid bacteria (LAB) on gastrointestinal health have been investigated in animal models and, occasionally, in human subjects. Some studies using yogurt, individual LAB species, or both showed promising health benefits for certain gastrointestinal conditions, including lactose intolerance, constipation, diarrheal diseases, colon cancer, inflammatory bowel disease, *Helicobacter pylori* infection, and allergies. Patients with any of these conditions could possibly benefit from the

consumption of yogurt. The benefits of yogurt consumption to gastrointestinal function are most likely due to effects mediated through the gut micro flora, bowel transit, and enhancement of gastrointestinal innate and adaptive immune responses (Adolfsson *et al*., 2004).

Yoghurt, curd or *Dahi* has nutritional benefits beyond those of original milk. The following points support the statement. *Dahi* is more palatable, and those who do not like drinking milk would consume it readily. *Dahi* is predigested food by microbial activity thus digested and assimilated more easily than milk. Lactose of milk is fermented into lactic acid, thus lactose intolerants can enjoy it. Moreover lactic acid solubilizes the calcium salts of milk which are more easily digested, assimilated and assist in bone formation.

A fermented dairy product from India, referred to as *Dahi*, has also been shown to have higher conjugated linoleic acid (CLA) content than does non-fermented *Dahi* (Aneja *et al*., 1990). The major sources of CLA in our diets are animal products from ruminants, in which CLA is synthesized by rumen bacteria. Increased consumption of dairy fat was shown to be associated with increased concentrations of CLA in both human adipose tissue and human milk (Jiang *et al*., 1999). CLA was reported to have immune-stimulatory and anti-carcinogenic properties. In a recent study of breast and colon cancer cells showed that the anti-carcinogenic properties of CLA may be due to the ability of some CLA isomers to inhibit the expression of cyclins and thus halt the progression of the cell cycle from G1to S phase. In addition, CLA induced the expression of the tumor suppressor p53 (Kemp *et al*., 2003).

Yoghurt also has medical uses, in particular for a variety of gastrointestinal conditions, and in preventing antibiotic-associated diarrhea. One study suggests that eating yoghurt containing *L. acidophilus* helps prevent vulvovaginal candidiasis. Some medical uses of Yogurt, from its wonderful antibacterial properties in the lactic cultures, have been clinically tested. Acidulates can be used successfully for:

a. Stopping dysentery caused by bacteria or by an unbalanced diet.

b. Regenerating the intestinal bacterial flora during and following antibiotic treatment.
c. Healing oral or skin infections such as eczema, ulcers and abrasions.
d. Soothing chronic constipation.
e. Helping gastro-intestinal problems.
f. Making up for a lack of vitamins (Five to ten percent more vitamin B12, riboflavin, phosphorus and potassium).
g. Feeding people with serious digestive problems
h. Substituting milk in cases of digestive or allergy problems
i. Alleviating states of anxiety.
j. Some yogurts contain active yogurt cultures that may lower blood pressure and prevent some cancers (Flora, 2004).

The lactic bacilli carry out some important and sometimes indispensable functions inside the intestinal bacterial flora such as:
a. Activating the digestion of glucosides and proteins.
b. The synthesis of group B (nervous balance and hepatic function) and group K (blood coagulation) vitamins.
c. Acidification of the intestinal tract thus preventing the development of pathogenic germs.
d. Synthesis of antibiotic substances (Adolfsson *et al*., 2004).

3.16 MICROORGANISMS OF *DAHI* OR YOGHURT

Microorganisms found in yoghurt can be divided in to two main groups

1. Lactic acid bacteria 2. Contaminant

Second group of microorganisms are foreign microbes or contaminate to *Dahi*. Their presence in yoghurt is harmful. These organisms are further divided into two groups,

A) Indicator organisms, those bacterial groups whose presence in any food above certain numerical level indicates poor sanitation, unhygienic handling and possibilities of presence of pathogenic organisms Such as coliforms (Hoadley, 1976). In dairy

science Staphylococci and *Geotrichum candidum* are also used as index or indicator organisms as the presence of former indicates handling abuse and the later, the machinery mold, as indicator of plant sanitation and contaminated equipments (Frazier and Westhoff, 2001).

B) Pathogenic microorganisms whose presence in food is undesirable and denouncing. They cause food borne diseases, food intoxication, abdominal discomforts and many diseases such as salmonella sp, Listeria sp, and Bacillus cereus etc (ICMSF. 1978).

Generally for microbiological quality specification of dairy products TCP, coliform count, Staphylococci count and detection of *Salmonella* is done (Hasell *et al*., 2001). Three types of microbiological criteria can be differentiated.

1. Microbiological Standard – a mandatory criterion that is included into a law or ordinance.
2. Microbiological Guideline – an advisory criterion used to inform food manufacturers on levels which can be achieved when applying best practices
3. Microbiological Specification – a part of a commercial agreement between a buyer and a supplier (Cordier, 2004).

3.17 LACTIC ACID BACTERIA

These bacteria are most abundant and load ranges to 2 millions per gram of quality yoghurt. They develop from starter and ferment the lactose of milk into lactic acid. This acidic condition clots the casein of milk to the characteristics of yoghurt. These microbes are essential for yoghurt making and come from starter. They also produce aroma, vitamins, and amino acids. Several mostly lactic and non lactic microbes in combination are used. These includes, symbiotic culture of *streptococcus thermophilus, lactobacillus delberki subsp bulgaricus, leuconostoc spp, bifidobacterium bifidus, lactobacillus acidophilus,* for acid production and *Streptoccous cremoris, S. diacetylactis S.lactics* and other harmless bacteria can be used for both acid and aroma

production (Frazier and Westhoff, 2001). A minimum of 10^7CFU/g of live and active characterizing cultures are required at the time of manufacture of yogurt (Frye, 2004; FDA, 1996).

3.18 CONTAMINANTS

Second group of microorganisms are foreign microbes or contaminate to yoghurt or curd. Their presence in yoghurt is harmful. These organisms are further divided into two groups; **A)** Indicator organisms, those bacterial groups whose presence in any food above certain numerical level indicates poor sanitation, unhygienic handling and possibilities of presence of pathogenic organisms Such as coliforms. **B)** Pathogenic microorganisms whose presence in food is undesirable and denouncing. They cause food borne diseases, food intoxication, abdominal discomforts and many diseases such as *salmonella sp, Listeria sp,* and *Staphylococcus sp* etc. The principal pathogens of concern associated with milk and processed milk products are *Salmonella* spp. *L. monocytogenes*, *S. aureus*, enterohemorrhagic *E. coli*, and Campylobacter *jejuni*, *C. botulinum*, B. *cereus*, *Mycobacterium tuberculosis*, *Shigella* spp. *Vibrio parahaemolyticus, haepatisis A and E viruses, Brucella spp* pathogenic *E. coli, Diptheria etc* (Frazier and Westhoff 2001).

3.19 YOGHURT'S MAIN ADULTERATING FACTORS

a. *Microbiological factors:* mould, yeast and bacteria are micro-organisms present in the environment, which cause food adulteration. Some of these, known as pathogenic, can cause illnesses and intoxications.

b. *Physical factors:* Light encourages the formation of free radicals, which can trigger degenerating phenomena. Heat encourages the development of germs.

c. *Chemical factors:* Oxygen allows food substances to oxidize. Enzymes are animal and vegetable cells own substances, which after the death of their host cause the destruction of the existing cell structures (Flora, 2004).

3.20 COMMON USES OF *DAHI* OR YOGHURT

For direct consumption, usually skimmed milk curd is used since it is low in fat and by the low-income group of the population because it is cheap yet nutritious. It can be consumed directly as beverage, or mixing with salts, sugars, or fruits as lasi or desserts for preparation of chakka, Sirkhanda, makkhan (butter). Mostly rural and margin farmers not having access for fresh milk sale adopt this method (Sukumar, 2002).

3.21 COMMON TYPES OF SPOILAGE OF *DAHI* OR YOGHURT OR CURD

3.21.1 Acidic or sour curd

Curd or *Dahi* has limited life time. After 0.5 to 1% of acid formation, it should be refrigerated to control further acid formation and thus souring. However prolonged incubation and inappropriate chilling may cause access acid formation and souring. Mixing of psychrophilic acid forming bacteria such as Alkaligenes, Micrococcus, pseudomonas, and coliforms can cause souring of curd in low temperature on long preservation (Frazier and Westhoff, 2001).

3.21.2 Gas productions

Gas production by bacteria is usually accompanied by acid formation and access gas production in yoghurt is undesirable. Chief gas formers are the coliforms bacteria, Clostridium spp., and gas forming *Bacillus* spp. that yields both carbondioxide and hydrogen. Yeasts, propionics and heterofermentative lactic acid bacteria produce carbondioxide only. Gas production in curd is evidenced by foam at the top, supersaturated with gas, by gas bubble caught in the curd or furrowing it, by floating the curd containing gas bubbles and is so called stormy fermentation (Frazier and Westhoff, 2001).

Gas development depends upon types of contaminating microbes, pretreatment, and temperature of holding. In pasteurized milk spores of Bacilli and Clostridia survive, later proliferate and accounts for major gas development.

3.21.3 Proteolysis

Proteolysis in milk is characterized by foul smell and bitter test and flavor. Proteolysis is favored by destruction of lactic bacteria or formed acid by heat or by milk moulds and yeasts films or neutralization of acid by product of other organisms. Proteolysis can be divided into:

1 acid proteolysis in which acid formation and proteolysis occur together and causes shrunken curd and the expression of much whey. This is followed by the slow digestion of curd, and may be completely dissolve by some kind of bacteria. Sometimes separate curd particles are formed that shrink to small that they are barely visible in large amount of whey. Acid proteolysis is mainly caused by several species of Micrococcus and *Streptococcus faecalis var liquefaciens* which is thermodeuric can survive to pasteurization and is the main bacteria to acidic proteolysis in pasteurized products (Sukumar, 2002).

2. Slow proteolysis by intracellular enzymes of bacteria after their autolysis is of no significance in milk under ordinary circumstances but is significant when along time is allowed for their action.

3. Sweet curdling *Bacillus cereus* has been implicated in sweet curling of milk. It happens due to proteolysis without acid formation.

4. Proteolysis with little acidity or even with alkalinity, proteolysis by bacteria unable to ferment lactose varies with the bacterium involved, from obvious digestion of casein to slightly proteolysis that is detectable only by chemical test. The product becomes alkaline as a result of protein decomposition. These bacteria include the *Micrococcus* spp., *Alcaligenes Pseudomonas, Proteus, Flavobacterium* and *Serritia* (Frazier and Westhoff, 2001).

3.21.4 Ropiness

Ropiness and sliminess can occur in milk curd or whey but are important in market milk and cream. Non bacterial ropiness or sliminess may be due to stiringiness caused by mastitis and in particular fibrin and leucocytes from cow's blood. In contrast to the ropiness produced by the bacteria, it is present when the milk is drawn, not developed during holding of the milk. Bacterial ropiness is caused by slimy capsular material from the cell usually gums or mucins and ordinarily develops based at low temperature storage. The ropiness usually decreases as acidity of curd increases. There are two main types of bacterial ropiness, one in which the curd most ropy at the top and the other in which the curd becomes ropy throughout. Surface ropiness is caused most often by Alcaligenes viscolyticus and organism chiefly from water or soil that can grow fairly well in the vicinity of 10^0C. Some of the thermoduric Micrococci e.g. *Micrococci freudenreichii* can cause the surface ropiness. Ropiness throughout the curd may be caused by *Enterococcus aerogenes, E. cloacae, Klebsiella oxytoca* and rarely *Escherichia coli*. Ropiness caused by the Enterobacter is worse near the top of the curd. Among the alkali formers Micrococci, certain strains of Streptococci and Bacilli produce ropiness throughout the curd. Adequate pasteurization of milk readily destroys most of these kinds of bacteria (Frazier and Westhoff, 2001).

3.21.5 Changes in Milk Fat

The fat content of *Dahi* may be decomposed by various bacteria yeasts and molds. The following changes in the *Dahi* fat takes place. Oxidation of unsaturated fatty acids which coupled with other decomposition, yields aldehydes, acids and ketones and results in the tallow odors and tastes. The reaction is favored by metals sunlight and oxidizing microorganisms. Hydrolysis of butter fat to fatty acids and glycerol by enzyme lipase. The lipase may have been in the original milk or may be microbial. Combined hydrolysis and oxidation activity cause production of rancidity.

Species of lipase forming bacteria are found in many bacterial genera e.g. *Pseudomonas, Proteus, Alcaligenes, Bacillus, Micrococcus, Clostridium* and others.

Many of the molds and some species of Yeast are also lipolytic. *Pseudomonas fragi* and *Staphylococcus aureus* produce fairly heat resistant lipases which may survive pasteurization if present in raw milk (Frazier and Westhoff 2001).

3.21.6 Alkali Productions

The group of alkali formers includes bacteria which cause the alkaline reaction in milk without any evidence of proteolysis. The alkaline reaction may result from the formation of ammonia, as from urea, or of carbonates, as from organic acids such as citric acid. Most of these bacteria grow from moderate to low temperatures, and many can survive the pasteurization. Examples of alkali formers are *Pseudomonas flurescens* and *Alcaligenes viscolactis* (Frazier and Westhoff 2001).

3.21.7 Flavor Change

Sour or acid flavor sour and acid flavor is produced by prolonged incubation or inappropriate temperature of incubation by the starters themselves. Refrigeration after appropriate acidity development can prevent this bad effect. Heterofermentative bacteria and Yeast are mainly responsible for sour flavor. Contaminants like coliforms clostridium species can produce sharp sour flavor.

Bitter flavor bitterness usually results from the proteolysis but may follow lipolysis or even fermentation of lactose. These organisms include coliforms, *Pseudomonas, Proteus, Micrococcus, Alcaligenes, Serratia, Bacillus cereus, B. subtilis* and some Yeast. Actinomycetes sometimes give bitter musty flavor. Burnt or caramel flavor certain strains of *Streptococcus lactis var maltigens* produce this flavor, which resembles the cooked flavor of overheated milk.

Miscellaneous other flavors these are less common flavor changes. Barny flavor by *Enterococcus oxitocum*, soapiness by ammonia formers by Pseudomonas sapolactica and turnip like flavor by *E. coli* and *Pseudomonas flurescens*, malty flavor by yellow Micrococci, fruity flavor by *Pseudomonas fragi*, fishiness by *Aeromonas hydrophilia*, alcoholic flavor by Yeast, an amyl alcohol flavor by white and orange Micrococci, and

putrefaction by species of *Clostridium*, *Pseudomonas putrefacien* and other putrefactive bacteria (Frazier and Westhoff 2001).

3.21.8 Color Changes:

The color of curd is affected by its physical and chemical composition. Color changes caused by the surface growth of pigmented bacteria or molds in the form of scum or ring or may be present throughout the curd. Blue curd: *Pseudomonas syncyanea* produces bluish gray to brownish color. When growing with an acid former like, *Streptococcus lactis* causes a deep blue color. *Actinomycetes* and *Geotricum* may produce blue color. Yellow curd *Psedomonas synxantha* may cause a yellow color in curd co-incident to lypolysis and proteolysis. Species of *Flavobacterium* may also give yellowness. Red curd is usually caused by species of *Serratia* e.g., *S. marcescens*. *Brevibacterium erythrogens* produce a red layer at the top of the curd, followed by proteolysis. *Micrococcus roseus* may grow and produce red sediment and yeast may produce pink or red colonies on the surface of sour curd. Brown curd a brown color may result from *P. putrefaciens* or by the enzymic oxidation of tyrosine by *p. fluorescens* (Frazier and Westhoff 2001).

3.22 MICROBILOGICAL QUALITY OF *DAHI* OR YOGHURT

The mean value of TPC, coliform, and yeasts and molds were outside the standard for all the dairy products which were analysed e.g. butter cheese yoghurt, ghee, cream, ice cream and paneer. The number of observations beyond the standard was also high for all these products. This indicates that the overall quality of dairy products were poor. The quality of yoghurt was analyzed in terms of total fat, total solids, acidity, coliform, and yeast and molds count and was found to be 2.69%, 16.42%, 1.18% 20832 cfu / ml and 3.4×10^7 cfu / ml. respectively (Waldhauer *et al*., 2001).

According to Diasty and Kaseh (2007), a total of 80 raw milk and yoghurt samples were randomly collected from different farm and retail markets of different sanitation level to be examined mycologically and bacteriological examination. Moulds and yeast detected in 80 % and 50 % of raw milk and yoghurt samples respectively, with mean values of

$4.3\times10^5 \pm 2.5\times10^5$ and $2.1\times10^4 \pm 1.9\times104$ cfu / ml, respectively. *Aspergillus* spp., *Cladosporium* spp., *Mucor* spp., *Curvularia* spp., *Penicillium* spp., *Geotricum* spp., *Candida* spp., *Rhodotorula* spp., *Torulopsis* spp. *and Saccharomyces spp*. isolated from both raw milk and yoghurt. While the mean Coliform count/ ml was $7.0\times10^6 \pm 5.0\times10^6$ and $6.0\times10^3 \pm 4.0\times10^3$ cfu / ml for raw milk and yoghurt respectively. But the mean enterobacteriaceae count /ml of raw milk and yoghurt samples $2.6\times10^6 \pm 5.5\times10^5$ and $1.5\times10^4 \pm 1.2\times10^4$.

The total viable mean count of *Dahi* was 7.34×10^7 cfu / ml with standard deviation of 1.57. The average coliform count of *Dahi* was 4.39×10^3 cfu per ml with standard deviation of 1.08. Coliforms were present in all samples, which reflect highly poor hygienic conditions and improper sanitation during manufacturing of *Dahi* (Younus *et al*., 2002).

Rashid *et al*, (2006) isolated a total of 266 strains of lactic acid bacteria (LAB) from 28 *Dahi* samples that were collected from different areas in Bangladesh. The isolated strains were identified on the basis of their morphological, physiological and biochemical characteristics. The cocci (73%) were dominant over the rods (27%). The distribution of the isolates by genus was as follows: *Streptococcus* (50%), *Lactobacillus* (27%), *Enterococcus* (9%), *Leuconostoc* (5%), *Lactococcus* (5%) and *Pediococcus* (4%). In this study, *S. bovis* was the most predominant species as this species represents 47.0% of the total isolates in *Dahi*. The other species they isolated were identified as *L. fermentum, L. delbrueckii* ssp. *bulgaricus, L. delbrueckii* ssp. *lactis, L.* ssp., *E. faecium, S. thermophilus, Leuconostoc mesenteroides* ssp. *mesenteroides, L. mesenteroides* ssp. *dextranicum, Lactococcus lactis* ssp. *lactis, L. raffinolactis* and *Pediococcus pentosaceus.*

Notermans *et al.* (1997) Due to the heat resistance of *Bacillus cereus*, its potential pathogenic character, the capability to grow in milk and reported diseases upon consumption of dairy products, the organism should be considered as hazardous in pasteurized milk. Human exposure experiments based on (1) enquiring about the storage

conditions (time and temperature) of pasteurized milk in households in The Netherlands and (2) performing of storage tests at 6, 8, 10 and 12°C, respectively, demonstrated the following: pasteurized milk is consumed within 2–12 days after pasteurization and is stored at temperatures varying from <5–13°C. Visually spoiled milk is not consumed anymore. Based on the distribution of both the storage time and temperature of milk in private households, approximately 7 and 4% of the total portions of milk consumed in The Netherlands could contain $>10^5$and $>10^6$ *B. cereus* ml^{-1}, respectively. Taking into account the high portion of milk consumed (approximately 10^9–10^{10}portions/year) and the absence of epidemiological evidence that milk is causing food borne diseases due to *B. cereus*, the dose–response relationship of this organism may need to be considered. Results of the storage tests, based on the worst case situation, provided useful information on the effect of the risk factors, storage temperature and time, to human exposure. However, such information can be obtained more easily by applying predictive models. Models also enable us to find out the initial number of spores which can be considered as a third risk factor.

In our investigation the 5 major food-borne pathogen groups, namely, *Salmonella* spp., *L. monocytogenes*, *C. jejuni*, and O157 and non-O157 STEC the major dairy borne pathogens for which dairy farms act as reservoirs or transient carriers (Murinda *et al*., 2004).

Rohrbach *et al*., (1992) reported that the frequency of isolation of foodborne pathogens from 292 bulk tank milk samples from dairies in east Tennessee and southwest Virginia was 12.3% *for C. jejuni*, 8.9% for *Salmonella* species, 4.1% for *L. monocytogenes*, and 15.1% for *Yersinia enterocolitica.* One or more foodborne pathogens were isolated from 32.5% of bulk tank milk samples evaluated

Introduction of *L. monocytogenes* into food processing plants results in reservoirs that are difficult to eradicate. For instance, biofilms are a constant issue in food processing environments. *Listeria monocytogenes* survived for extended periods on stainless steel and buna-n rubber, materials commonly used in food-processing equipment (Wong,

1998). In a study conducted in 21 dairy processing plants in Vermont, 80 of 378 sites (21.2%) were identified as *Listeria*-positive and of these, 35 (43.8%) were positive for *L. monocytogenes* (Pritchard *et al*., 1995). 68 of 195 (34.9%) dairy producers in East Tennessee and Southwest Virginia consumed raw bulk tank milk produced on their farm. Of the bulk tanks from which raw milk was consumed by dairy producers, 25% (17 of 68) contained one or more species of *L. monocytogenes*, *C. jejuni*, *Y. enterocolitica* and *Salmonella* (Rohrbach *et al.,* 1992).

The high frequency of *Campylobacter* isolation in feces of cows suggests that contamination of bulk tank milk occurs primarily via feces of *Campylobacter*-carrier cows during harvesting and/or storage of raw milk (Waterman *et al.,* 1984).

In the study by Saravanakumar *et al*., (2007) *Penicillium* spp, 43.4%of samples, *Aspergillus* spp in 36.7% of samples were detected and common molds were Fusarium spp, Cladosporium spp, Mucur spp, *Rhizopus* spp. Out of 10 *Aspergillus ochraceus* isolates obtained from curd, only four isolates were able to produce ochratoxin in the artificial medium. The yeasts load were 1.92×10^6, 1.58×10^5 and 1.15×10^5 cfu/m for *Dahi* of bulk vendors, hotels and households respectively.

3.23 SOME COMMON MILK BORNE PATHOGENS AND DISEASE CAUSED BY THEM

Pathogenic organisms of both bovine and human origin have been isolated from milk. Milk and milk products, therefore, can serve as the carrier of different diseases. Many serious epidemics were caused by consumption of such products before this fact was clearly recognized. However, this became less common as milk sanitation has improved and pasteurization is being more widely practiced.

The health of animal is important factor. Several diseases of cattle including *Staphylococcus* and *Streptococcus* infections, tuberculosis, brucellosis, salmonellosis, Q fever and foot and mouth disease may be transmitted to man. Some of the important diseases of human origin that have been transmitted by milk are (1) typhoid fever (2)

diphtheria (3) scarlet fever (4) dysentery (5) septic sore throat and (6) poliomyelitis. It is also possible to infect animals. For example, mastitis may be caused by a variety of organisms, including *Staphylococcus aureus*. The infecting organism, in some case, has been traced to human (Frazier and Westhoff, 2001).

3.23.1 Staphylococcus Food Intoxication

One of the most commonly occurring food poisoning is caused by the ingestion of the enterotoxin formed in the food during growth of certain stains of Staphylococcus aureus. The toxin is termed an enterotoxin because it causes gastroenteritis or inflammation of the lining of the intestinal tract. However, not all coagulase positive Staphylococci are necessarily enterotoxigenic. The organism is fermentative, poteolytic, high salt and fairly dissolved sugar (50 to 60 percent of sucrose) tolerant and don't produce obnoxious odor in most food or make them appear unattractive. Staphylococcal food poisoning may encounter to us repeatedly in life time and are usually not reported or publicized until the out break is fairly large or severe as in picnic, large diner, party, and convention. Milk and milk products are common food for staphylococcal food poisoning as they highly promote the growth and toxin production and also have chance of contamination during handling and processing.

The most common human symptoms are salivation, then nausea, vomiting, retching, abdominal cramping of varying severity and diarrhea. Blood and mucus may be found in stool and vomits in severe cases. Headache, muscular cramping, sweating, chills, prostration, weak pulse, shock, and shallow respiration may occur. Usually subnormal body temperature is observed rather than fever. The mortality is extremely low (Frazier and Westhoff, 2001).

3.23.2 Salmonellosis

Salmonellosis may result following the ingestion of viable cells of a number of genus Salmonella. It is severe and most frequent bacterial food borne infection. There are several species of salmonella to cause Salmonellosis which are over a thousand known serovars. The Salmonella infections that are called food poisoning may be caused by

any of a large number of serovars e.g. *Salmonella choleraesuis, S. enteritidis, S. typhimurium S. heidelberg. S. derby, S. infantis S. montevideo*, etc. Salmonella are the member of family enterobacteriaceae which are gram negative rods capable of glucose fermenting with gas but not lactose and sucrose. Milk contains almost all nutrients for growth factors for proliferation for Salmonella and milk borne Salmonellosis is frequently reported. Salmonella contamination usually occurs during handling and processing due to contaminated utensils, hands, and fecal contaminated water. *Salmonella typhimurium* is the main milk borne Salmonellosis and gastroenteritis and other may also occur and cause the disease.

Usually million for most infective strain and hundred of million to billion of organisms should be ingested for Salmonellosis and incubation period is about 12 to 36 hours. The main symptoms of Salmonella gastrointestinal infection are nausea, vomiting, abdominal pain, and diarrhea that usually occur suddenly. This may be preceded by headache and chills. Other evidences of diseases are watery, greenish foul smelling stool, prostration muscular weakness, faintness, usually moderate fever, restlessness, twitching and drowsiness. If treatment is given in proper time, mortality is very few about less than one percent. Proper pasteurization and prevention of post pasteurization contamination by good sanitary condition and hygienic practice prevents the incidence of Salmonellosis (Brooks *et al*., 2004).

3.23.3 Enteropathogenic *Escherichia coli*

E. *coli* is generally regarded as part of human normal flora of intestinal tract and that of many animals. Several nursery epidemics and human diarrhea disease and food poisoning E. coli have been designated as Enteropathogenic *E. coli* (EEC). The human disease syndromes resulting from the ingestion of EEC have been divided in two main groups. The first group consists of strains which produce an enterotoxin and result in a cholera like or enterotoxigenic illness in humans. The enterotoxigenic strains usually produce two enterotoxins, a heat stable (ST) and heat labile (LT) toxin and are thought to be responsible for infantile diarrheal diseases and travelers diarrhea. To experience

the enterotoxigenic illness, EEC serotypes capable of elaborating the enterotoxins must be ingested, followed colonization in the upper small intestine and production of enterotoxin. The enterotoxins mediate the net movement of water into the intestinal lumen in the absence of invasion, penetration and without any macroscopic change in the intestine.

Second major group consist of invasive strains which produce cytotoxin and result in the invasive illness, colitis, and dysentery like syndrome. These serotypes are non enterotoxigenic, grow in the colon and invade or penetrate the epithelial cells of colonic mucosa. *E. coli* are common food contaminating organisms. It is also taken as indicator organism of fecal contamination of foods. Bad sanitation, personal unhygienic practices and mixing of fecal contaminated water in milk are the main cause of *E. coli* contamination. Milk promotes the growth of contaminated organisms if the proper preservation methods such as cooling and refrigeration are not applied (Frazier and Westhoff, 2001).

3.23.4. *Bacillus cereus*

The genus bacillus includes large aerobic, gram positive rods occurring in chain. These organisms are most prevalent organisms of environment in soil, foods, water, and air. Bacillus cereus can grow in foods and produce an enterotoxin or an emetic toxin and cause food poisoning. The food poisoning caused by *Bacillus cereus* has two forms, the emetic type, and diarrheal type. *Bacillus cereus* produces toxins that cause disease that is more an intoxication than a food-borne infection. The emetic form is manifested by nausea, vomiting, abdominal cramping, and occasionally diarrhea, and is self limiting with the recovery within 24 hours. *Bacillus cereus* contamination in milk and dairy product is common as it is spore former can withstand pasteurization and post pasteurization contamination is common being common environment bacteria (Power, Dajinawala, 2001).

3.23.5. Tuberculosis

Milk borne tuberculosis is caused by organism *Mycobacterium bovis* and the symptoms are similar to the disease caused by *Mycobacterium tuberculosis*. Disease is communicated by consumption of improperly pasteurized or boiled milk from infected cattle. This organism is closely related to *M. tuberculosis*. Due to implementation of pasteurization the disease incidence is dramatically reduced (Brooks *et al.,* 2004).

3.23.6. Brucellosis

Brucellosis is a true zoonosis, primarily affecting domestic animals goats, sheep, cattle, buffaloes, pigs. Human beings are secondarily infected. It is early recognised in meditarian region and is known as mediterian fever, malta fever, or undulant fever. *B.melitensis, B. abortus, B. suis* are 3 major species causing muman brucellosis. This disease is world wide in distribution and endemic in certain region like mediterian *(B.melitensis), B. abortus* in Great Britain, *B. suis* in USA. It is caused by consumption of improperly pasteurized milk of various cattle especially goat. The infected cattles excrete the organism in milk for years. The pasteurisation of milk, pruning of diseased herds, and vaccination of cattles by attenuated *B. abortus* has resulted in spectacular fall in the incidence of human brucellosis (Frazier and Westhoff, 2001).

3.23.7. Shigellosis

Shigellosis is also called bacillary dysentry and is common food borne disease of developing countries. It is caused by seveal species of genus *Shigella, e.g. Shigella sonnei, S. flexneri, S. dysenteriae, S. boydii*. Incubation period is 1-7 days and usually less than 4 days. Clinical symptoms are extremely variable from mild to severe depending upon the type of strain, ingestion dose, immune status of individuals. Abdominal cramps, fever etc. (Frazier and Westhoff, 2001).

3.23.8. *Listeria monocytogenes*

In the past 25 years, Listeria monocytogenes has become increasingly important as a food-associated pathogen (Farber, 1991). Most European Union countries have an annual incidence of human listeriosis of between two and ten reported cases per million.

Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness. Listeria monocytogenes infections are responsible for the highest hospitalisation rates (91%) amongst known food-borne pathogens and have been linked to sporadic episodes and large outbreaks of human illness worldwide (Mead, 1999). The ability to persist in food-processing environments and multiply under refrigeration temperatures makes *L. monocytogenes* a significant threat to public health (Walker, 1990). *Listeria monocytogenes* contamination is one of the leading microbiological causes of food recalls, mainly of meat, poultry, and seafood and dairy products (Schuchat *et al*, 1991).

Prevention and control measures are based on hazard analysis and critical control point programmes throughout the food industry, and on specific recommendations for high-risk groups. Understanding how these micro-organisms adapt their cellular physiology to overcome stress is important in controlling L. monocytogenes in food (Husu, 1990).

3.23.9. *Yersinia enterocolitica*

This common organism has been found in many foods of animal origin including milk, cheese, and red. *Yersinia*, found in streams, lakes, and wells, spreads from the water to warm-blooded animals. The most common symptom of yersinosis is gastroenteritis and mimics the symptoms of appendicitis. *Yersinia enterocolitica* is destroyed by pasteurization. (Smith 1981)

3.23.10. *Campylobacter jejuni*

This organism, isolated in raw milk and meat, can cause mastitis in dairy cattle. It has also been isolated in the feces of many species including dogs, cats, rodents, cattle, sheep, swine, and poultry. Symptoms include vomiting, cramps, bloody diarrhea, mild enteritis, or severe enterocolitis. Individuals who have recovered from the disease may suffer a relapse. *Campylobacter jejuni* is destroyed by pasteurization. (Smith 1981)

3.24. THE FUNGI COMMONLY ISOLATED AS CONTAMINANT IN *DAHI* OR YOGHURT

3.24.1. *Rhizopus* spp.

It is a fast growing, terrestrial fungus. Hyphae are coenocytic, aseptate and asexual reproduction by non motile sporangiospores and sexual reproduction by formation of zygospores. There is presence of stolons and darkly pigmented rhizoids. The disease typically involved in the rhino faciocranial area, lungs, gastrointestinal tract and skin (Collier *et al*., 1998).

3.24.2. *Fusarium* spp.

It is a filamentous, hyaline with septate hyphae, conidiophores, phialides, macroconidia, and microconidia are observed microscopically, chlamydospores are also produced. Phialides are cylindrical, with a small collarette, solitary or produced as a component of a complex branching system (Collier *et al*., 1998).

It is opportunistic and causative agent of superficial and systemic infections in humans; fusariosis, keratitis, endophthalmitis, otitis media, onychomycosis, cutaneous infections particularly of burn wounds, mycetoma, sinusitis, pulmonary infections, endocarditis, peritonitis, central venous catheter infections, septic arthritis, and fungemia (Collier *et al*., 1998).

3.24.3. *Aspergillus* spp.

It is filamentous, with branching hyaline and septate hyphae and usually multinucleated reproduce by means of asexual spores termed conidia; produce in basipetal fashion.

It causes allergic aspergillosis, infection of the paranasal sinuses, sinusitis, and aspergilloma, infection of the central nervous system, ocular infection, endocarditis, myocarditis, osteomyelitis, otomycosis, and infection of skin (Collier *et al*., 1998). Many species of Aspergillus produce potent mycotoxin and cause food intoxication (Frazier, 2001).

3.24.4. *Geotricum* spp.

It is yeast found worldwide in soil, water, cereals and dairy products, in normal human flora and is isolated from sputum and feces. It produces rapidly growing, white, dry, powdery to cottony colonies. Arthroconidia ("disjunctor cells") and coarse true hyphae are observed (Larone, 1995).Geotricum candidum (Oospora lactis) is common Dairy contaminants and is also known as dairy molds and is the indicator of dairy plant machinery sanitation(Frazier and Westhoff 2001).

It causes opportunistic infections referred to as geotrichosis. Bronchial and pulmonary as well as disseminated infections and fungemia due to Geotrichum have been reported (Buchta and Otcenasek, 1997).

3.24.5. *Mucor* spp.

Mucor is common molds occurring in dairy products and involves in spoilage. It is widely distributed in nature and is also used in manufacture of amylase enzymes and oriental foods (Frazier and Westhoff 2001).

3.24.6. *Cladosporium* spp.

These dark molds cause black spot on a number of foods. Colonies of C. herbarum are restricted in growth and are thick, velvety, and olive to grey-green. The reserve side of plant is a stricking opalescent blue to greenish-black (Frazier and Westhoff 2001).

3.24.7. *Penicillium* spp.

It species are widespread in nature and important in food. Many of them produce potent mycotoxins and food poisoning. Some species are used in manufacture of food such as cheese (Frazier and Westhoff 2001).

CHAPTER-IV

4. MATERIALS AND METHODS

The study was carried out at Central Department of Microbiology, Kritipur, Kathmandu Nepal since July 2007 to Jan 2008 for six months. The study was designed to evaluate the microbiological quality of *Dahi* of dairy industries with higher product capacity. A total of 71 samples from major locations of three districts of Katmandu valley were collected and laboratory analysis for microbiological quality was done in CDM laboratory.

4.1 MATERIALS

List of all the materials used in this study are given in appendix-II.

4.2 METHODS

4.2.1 Sampling Sites

Altogether 11 sampling sites, Baneshwor, Koteshwor, Maharajgunj, Patan, Sanothimi, Bhaktapur, Kalanki, Krtitipur, Gangabu, Lainchaur and Ratna park area were selected for sample collection. During sample collection only relatively large industries with higher capacity of production were selected though the large numbers of *Dahi* manufacturing industries were available.

4.2.2 Sample Collection

Sample collection was done aseptically using sterile materials, instruments and containers. A short questionnaire was taken from dairy owners about us of starters, process of manufacturing, preservation, selling and storage practice. The samples were immediately carried to the laboratory of Central Department of Microbiology, Tribhuvan University Kritipur, Kathmandu. Usually laboratory analysis was done in the same day otherwise the samples were refrigerated at 4^0C for preservation and laboratory analysis was done next day.

4.2.3 Sample processing

All the collected samples were processed and analyzed according to standard methods prescribed by ICMSF (1986) and DFTQC (2002).

4.2.4 Preparation of sample

As soon as the samples were delivered to laboratory, preparation of sample for laboratory analysis was done. The curd samples were vortexed and mixed well to form uniform and smooth suspension.

4.2.5 Serial dilution of *Dahi* samples

Well mixed 25ml *Dahi* samples were poured in the conical flasks and the volume was made 250 ml by adding sterile normal saline. It was again well shaken for uniform distribution of sample. It is 10^{-1} dilution. One ml of the 10^{-1} diluted sample was pipetted out in to the10^{-2} labeled tube containing 9 ml of sterile normal saline which was10^{-2} dilution which was well shaken. From the second dilution, 1ml was transferred into third10^{-3} labeled tube containing 9 ml of sterile normal saline which was 10^{-3} dilution and well shaken. This process was done repeatedly up to 10^{-6} dilution.

4.2.6 Microbiological analysis

The prepared sample i.e. serially diluted sample was subjected to microbial analysis. During the study period total coliform count, Staphylooccal count, yeast and mold count, *Bacillus cereus* count and detection of *Salmonella* and *Shigella* were carried out.

4.2.7 Procedure

4.2.7.1 Total coliforms count

For the enumeration of total coliforms 10^{-1} to 10^{-3} diluted samples were used. One ml of diluted samples were pipetted out to the respective plates and melted lukewarm VRBA media was poured and the plates were rotated gently for uniform distribution of samples in the media. The plates were incubated at 37^{0}C for 24 hours. After incubation the enumeration of colonies was done. The isolation was carried out on NA for different colonies for identification.

4.2.7.2 Staphylococci count

For the enumeration of Staphylococci, 10^{-1} to 10^{-3} diluted samples were taken. 0.1 ml of diluted samples were pipetted out to on the surface of MSA and spreaded with sterile glass rod.The plates were incubated at 37^0C for 24-48 hours. After incubation observation was carried out for mannitol fermenting colonies. The isolated Staphylococci were subcultured on NA for identification.

4.2.7.3 *Bacillus cereus* count

Counting of *Bacillus cereus* was done in Polymyxin Egg emulsion Mannitol Bromothymol blue agar (PEMBA) media. For enumeration, 10^{-1} to 10^{-3} diluted samples were used. Pour plate method was applied.The plates were incubated at 37^0C for 24 hours. After incubation observation was carried out for mannitol fermenting *Bacillus cereus* specific colonies (white colonies with blue surroundings). The isolated *Bacillus cereus* was subcultured on NA for identification.

4.2.7.4 Fungal count

For the enumeration of Yeast and molds, 10^{-3} to 10^{-5} diluted samples were taken. 0.1 ml of diluted samples were pipetted out to on the surface of PDA (incorporated with Chloramphenicol) and spreaded with sterile glass rod. The plates were incubated at 28^0C for 1 to 7 days. Yeast count was done after 24 hours of incubation and fungi were counted after 5 to 7 days. Molds were subcultured on PDA for identification.

4.2.7.5 *Streptococcus thermophilus* count

M_{17}, the selective media for *Streptococcus thermophilus* was used for the enumeration. Pour plate method for 10^{-1} to 10^{-3} diluted samples was used for counting. The plates were incubated at 37^0C for 24 hours. The isolated colonies were subcultured on NA for identification.

4.2.7.6 Isolation of *Salmonella* and *Shigella* spp.

Pre-enrichment:-The well mixed and emulsified *Dahi* was aseptically transferred into a conical flask containing 500ml of buffered peptone water (BPW) and incubated at 37^0C for 24 hours.

Enrichment One ml of the pre-enriched culture was aseptically transferred to test tubes of selenite –F broth, and incubated at 37^0C for 24 hours for enrichment of *Salmonella* and *Shigella.*

Plating out: - After 24 hours, a loopful of the enriched culture was streaked on SS agar and incubated at 37^0C for 24 hours. Next day the palates were observed for growth and subcultured was carried out on NA for identification.

4.2.8 Identification of the Organisms

4.2.8.1 Identification of bacteria

Identification of all the bacteria were done by using microbiological techniques as described in the Bergey's manual which involves morphological appearance of the colonies, staining reactions and biochemical properties. Standard protocol provided by Cheesbrough (1984) and Collee *et al.* (1996) was followed for identification of bacteria. The method of preparation of media, reagents and inoculation of biochemical media is described in appendix-XIII.

4.2.8.2 Identification of Fungi

Fungi were identified by colonial characteristics and microscopy of mycelia and shape and arrangements of spore in lactophenol cotton blue staining as described by Cheesbrough (1984).

4.2.9 Data analysis

The data were analyzed by using SPSS 11.5 version and Microsoft Excel 2007. The results obtained were analyzed by ANOVA test. The test of this study is mentioned in appendix-VII.

CHAPTER-V

5. RESULTS

During the study period July 2007 to January 2008, seventy-one *Dahi* samples were collected from ten major locations of three districts of Kathmandu valley and processed according to standard microbiological procedure at research laboratory of Central Department of Microbiology, Kathmandu.

5.1 SURVEY REPORT

5.1.1 Storage Practice

The storage practices of *Dahi* being practiced by the sellers were also monitored and found that most of the sellers adopted shelf storage practice followed by refrigeration and on floor. The results are shown in figure 1.

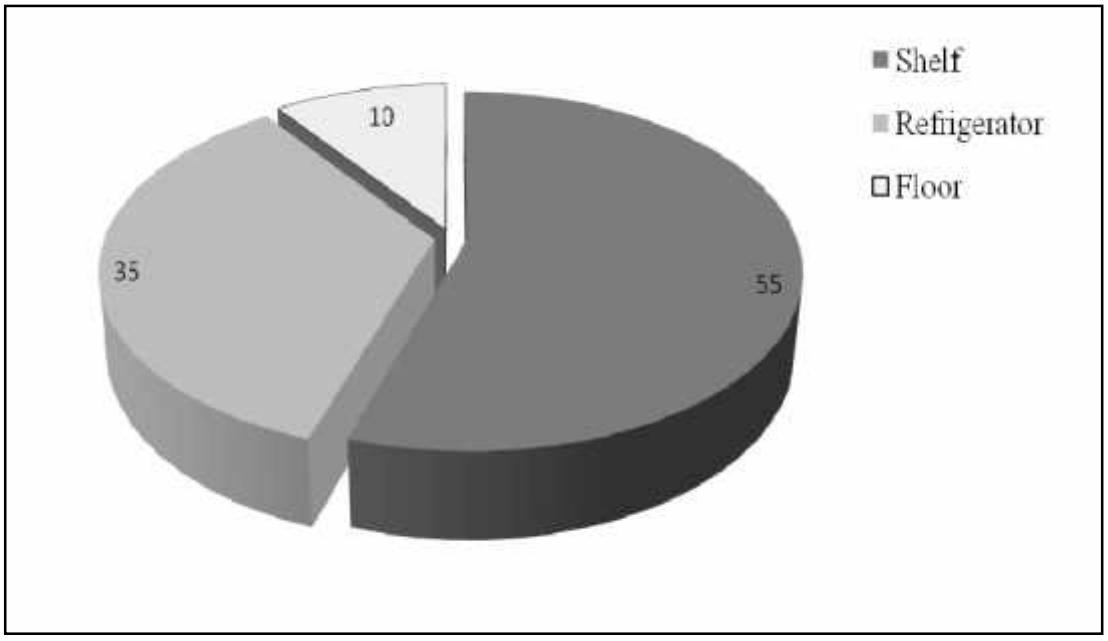

Figure 1: Percentage of storage practice of *Dahi*

5.1.2 Selling Practice

The sellers were found to use plastic cups, ceramic cups and plastic bags for packaging of *Dahi* during selling. Most company has adopted more than two methods of selling practices. The following table depicts the percentage of selling practices of *Dahi* collected for analysis. The results are shown in figure 2.

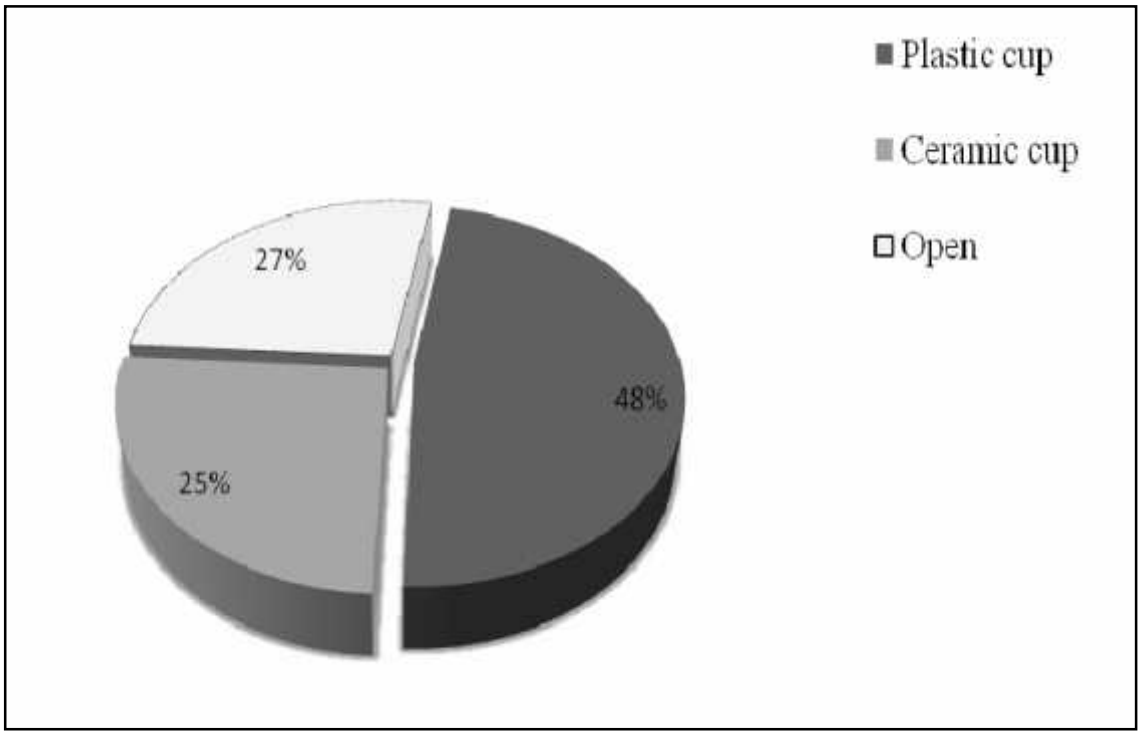

Figure 2: Percentage distribution of containers used in *Dahi* selling practice

5.2 BACTERIOLOGICAL ANALYSIS OF *DAHI*

5.2.1 Level of *Streptococcus thermophilus* in *Dahi*

Among the 71 samples only 3 (4.23%) were positive for Streptococcus *thermophilus* with mean count of 2.57×10^5 cfu / ml. All the *S. thermophilus* were found to be urease deficient. The results are shown in table 4.

Table no. 4: Level of *Streptococcus thermophilus* in *Dahi*

Positive samples	Negative samples	Positive %	Negative %	Mean count	Urease
3	68	4.23	95.77	2.57×10^5	-ve

5.2.2 Level of total coliform in *Dahi*

Among seventy-one samples, 32(45.08%) samples were positive for coliform. The mean coliform count was found to be 1.48×10^4 cfu / ml. The highest count was 3.41×10^5 cfu / ml and the lowest was 66 among the coliform positive samples. The results are shown in table 5.

Table 5: Level of total coliform in *Dahi* samples

S.N.	Range (cfu / ml)	No. of sample	% of sample	Mean (cfu /
1.	ND	39	54.92	1.48×10^4
2.	$1\text{-}10^2$	1	1.40	
3.	$10^2\text{-}10^3$	10	14.08	
4.	$10^3\text{-}10^4$	6	8.45	
5.	$10^4\text{-}10^5$	13	18.34	
6.	$10^5\text{-}10^6$	2	2.81	
Total		71	100	

ND= not detected

5.2.3 Level of Staphylococci in *Dahi* samples

Sixty-six (92.95%) of seventy-one samples revealed Staphylococci. The mean Staphylococci count was found to be 3.18×10^4 cfu / ml. The highest count was 4.69×10^5 cfu / ml and the lowest was 33 cfu / ml among the Staphylococci positive samples. The results are shown in table 6.

Table 6: Level of Staphylococci in *Dahi* samples

S.N.	Range (Cfu / ml)	No. of sample	% of sample	Mean (cfu /
1.	ND	5	7.05	
2.	$1\text{-}10^2$	4	5.64	
3.	$10^2\text{-}10^3$	9	12.67	
4.	$10^3\text{-}10^4$	16	22.53	3.18×10^4
5.	$10^4\text{-}10^5$	33	46.47	
6.	$10^5\text{-}10^6$	4	5.64	
Total		71	100	

ND= not detected

5.2.4 Level of fungi in *Dahi* samples

Almost all samples 98.60% (70/71) were found to contain Yeast and mold. The mean Yeast and mold count was found to be 2.5×10^7 cfu / ml. The highest count was 1.07×10^8 cfu / ml and the lowest was 5.16×10^5 cfu / ml among the Yeast and mold cotaining samples. The results are shown in table 7.

Table 7: Level of fungi in *Dahi* samples

S.N.	Range (Cfu / ml)	No. of sample	% of sample	Mean (cfu / ml)
1.	ND	1	1.40	2.5×10^7
2.	10^5-10^6	2	2.81	
3.	10^6-10^7	15	21.15	
4.	10^7-10^8	52	73.24	
5.	10^8-10^9	1	1.40	
Total		71	100	

ND= not detected

5.2.5 Level of *Bacillus cereus* in *Dahi* samples

88.73% of samples were positive for *Bacillus cereus*. The mean *Bacillus cereus* count was found to be 5.51×10^5 cfu / ml. The highest count was 2.99×10^6 cfu / ml and the lowest was 4×10^3 cfu / ml among the *Bacillus cereus* positive samples. The results are shown in table 8.

Table 8: Level of *Bacillus cereus* in *Dahi* samples

S.N.	Range (Cfu / ml)	No. of sample	% of sample	Mean (cfu / ml)
1.	ND	8	11.27	5.51×10^5
2.	10^3-10^4	4	5.64	
3.	10^4-10^5	9	12.67	
4.	10^5-10^6	36	50.71	
5.	10^6-10^7	14	19.71	
Total		71	100	

ND= not detected

5.2.6 Type of vessel and mean microbial load in *Dahi*

Evaluation of microbial load was done on the basis of type of vessel used to sell *Dahi*. Staphylococcal and yeast and mold count were maximum in plastic cup *Dahi* whereas Coliform and *Bacillus cereus* count were maximum in ceramic cup *Dahi*. The results are shown in table 9.

Table 9: Type of vessel and mean microbial load in *Dahi*

S.N.	Type of container	Mean (cfu / ml)			
		Coliform	Yeast	Staphylococci	*Bacillus cereus*
1.	Plastic cup	1.38×10^4	2.7×10^7	7.63×10^4	5.62×10^5
2.	Ceramic cup	2.18×10^4	2.2×10^7	4.38×10^4	7.29×10^5
3.	Open	9.94×10^3	2.5×10^7	1.11×10^4	3.61×10^5

5.2.7 Pattern of microbial isolates isolated from *Dahi*

Escherichia coli 96.87% (31/32) was the most frequently encountered coliform which was followed by *Klebsiella pneumoniae* 46.87% (15/32). Other coliforms detected were *Citrobacter freundii* (6.25%) and *Enterobacter aerogenes* (6.25%). Only one sample (1/71) was positive for *Salmonella* and identified as *Salmonella typhimurium.* Both coagulase positive and coagulase negative Staphylococci were present in 78.78% (52/66) samples. Coagulase positive and negative Staphylococci were 87.87% (66/71) and 90.90% (60/66) respectively.

Most common fungi were found to be Yeast 98.60% (70/71) which was followed by *Geotrichum* spp. 49.20% (31/63). Molds detected were *Aspergillus* spp. (44.44%), *Mucor* spp. (42.85%), *Penicillium* spp. (31.74%), *Cladosporium* spp. (7.93%), *Fusarium* spp. (9.52%) and *Rhizopus* spp. (6.34%). The results are shown in table 10.

Table no.10: Pattern of microbial isolates isolated from *Dahi*

S.N.	Organism identified	No. of samples positive for	No. of samples			
			Positive		Negative	
			No.	%	No.	%
1.	*Escherichia coli*	Coliform (N=32)	31	96.87	1	3.13
2.	*Klebsiella pneumoniae*		15	46.87	17	53.13
3.	*Citrobacter freundii*		2	6.25	30	93.75
4.	*Enterobacter aerogenes*		2	6.25	30	93.75
5.	*Salmonella* spp.	N=1	1	1.40	70	98.60
6.	Coagulase positive Staphylococcus	*Staphylococcus* (N=66)	58	87.87	8	12.13
7.	Coagulase negative Staphylococcus		60	90.90	6	9.10
8.	Samples with both types of Staphylococci		52	78.78	14	21.22
9.	*Aspergillus* spp.	Yeast and Molds (N=70)	28	40.00	42	60.00
10.	*Mucor* spp.		27	38.57	43	61.43
11.	*Penicillium* spp.		20	28.57	50	71.43
12.	*Cladosporium* spp.		5	7.14	65	92.86
13.	*Geotrichum* spp.		31	44.29	39	55.71
14.	*Fusarium* spp.		6	8.57	64	91.43
15.	*Rhizopus* spp.		4	5.71	66	94.29
16.	*Bacillus cereus*	N=63	63	88.73	8	11.27
17.	*Streptococcus thermophilus*	N=3	3	4.23	68	95.77

5.2.8 Statistical Analysis: The difference in occurrences of the pathogens and spoilage organisms on the basis of type of container was found but it was statistically (ANOVA) insignificant.

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1. DISCUSSION

Milk and milk products are a rich and convenient source of nutrients for people in many countries and international trade of milk-based commodities is significant. General as well as specific code should be developed to provide guidance to ensure the safety and suitability of milk and milk products to protect consumers' health and to facilitate trade. Code should provide guidance to countries so that their appropriate level of public health protection for milk and milk products may be achieved. Deliberately developed codes prevent unhygienic practices and conditions in the production, processing and handling of milk and milk products (Codex, 2003).

All foods have the potential to cause food borne illness; milk and milk products are no exception. Dairy animals may carry human pathogens. Such pathogens present in milk may increase the risk of causing food borne illness. Moreover, the milking procedure, subsequent pooling and the storage of milk carry the risks of further contamination from man or the environment or growth of inherent pathogens. Further, the composition of many milk products makes them good media for the outgrowth of pathogenic microorganisms. Potential also exists for the contamination of milk with residues of veterinary drugs, pesticides and other chemical contaminants. In many countries milk and milk products form a large portion of the diet of consumers especially infants, children, and pregnant and lactating women. Therefore, implementation of the proper hygienic control of milk and milk products throughout the food chain is essential to ensure the safety and suitability of these foods for their intended use (FAO, 2004).

In this study, total of 71 *Dahi* samples from three districts of Kathmandu valley were collected for microbiological quality evaluation. 34 plastic cups, 19 open and 18 ceramics cups *Dahi* were processed in the laboratory. For bacteriological quality assessment both indicator organisms e.g., fecal coliforms and food-borne pathogenic organisms like *Bacillus cereus, Staphylococci*, *Salmonella* and *Shigella* were studied Enumeration of yeasts and molds and isolation and identification of moulds were done for mycological quality assessment.

Streptococcus thermophilus is a commom starter of yoghurt. It is gram positive occuring in pairs to short chain, a lactic acid thermodeuric bacterium (Frazier, 2001). Its count in *Dahi* indicates the use of standard starter and low contamination because it ferments the sugar of milk first creating the highly acidic condition so that the proliferation of contaminating spoilage and pathogenic organisms can't take place. It is universal starter of yoghurt and yoghurt like fermented milk products which are known by different name all over the world (Lanyi, 1987). In this study only very few samples (4.23%) contained *Streptococcus thermophilus*. Some large dairy companies of Kathmandu use exotic starters and thus their *Dahi* contain *Streptococcus thermophilus*. Their count is also satisfactory with mean count of 2.57×10^5 cfu / ml. This result is different from Rashid *et al*. (2006) they isolated *Streptococcus* (50%) from different market *Dahi* samples in Bangladesh.

Most of the wild strains of *Streptococcus thermophilus* contain the urease enzyme which hydrolyses the urea and some amino acids with production of ammonia. Thus produce ammonia interfere with regulity of acidity, bitter taste and thus the quality. Urease deficient strain of *S. thermophilus* is used in the commercial dairy industries for better quality of fermented dairy products in which *S thermophilus* is used as starter (Mora *et al.*, 2002).

In present study all the isolates of *S. thermophilus* were found to be urease deficient. For the identification of organism Staining microscopic characteristics and carbohydrate

fermentation tests were applied. Most of the local *Dahi* contained the high load of *Streptococcus faecalis* but not *Streptococcus thermophilus*. *Streptococcus faecalis* is the indicator of faecal contamination and poor sanitation (Krieg *et al*., 2001).

Among 71 samples, 32(45.08%) samples contained coliform with mean count 1.48×10^4 cfu / ml. The highest count and the lowest count was 3.41×10^5 cfu / ml and 66 cfu / ml indicating inconsistent coliform load in the samples. The maximum samples 18.34% (13/71) were in the range of 10^4-10^5 cfu / ml. This somehow resembles with the report where 79.40% (40/51) samples were coliform positive, mean count 2.08×10^4 cfu / ml with maximum and minimum count 3.01×10^5 and 3cfu / ml respectively and maximum samples 25.49% (12/51) were in the range of 10^4-10^5 cfu / ml (Waldhauer *et al.*, 2001). Similar study done by El-Diasty and El-Kaseh (2007) in Jordan showed the mean Coliform count/ ml $7.0\times10^6 \pm 5.0\times10^6$ and $6.0\times10^3 \pm 4.0\times10^3$ cfu / ml for raw milk and yoghurt respectively. But the mean Enterobacteriaceae count /ml of raw milk and yoghurt samples $2.6\times10^6 \pm 5.5\times10^5$ and $1.5\times10^4 \pm 1.2\times10^4$ cfu / ml. The total viable mean count of *Dahi* was 7.34×10^7 cfu / ml with standard deviation of 1.57. This study somehow is in agreement with Younus *et al*. (2002) in which average coliform count of *Dahi* was 4.39×10^3 cfu per ml with standard deviation of 1.08. Coliforms were present in all samples, which reflect highly poor hygienic conditions and improper sanitation during manufacturing of *Dahi*.The mean coliform count is very high alarming the microbiological quality of *Dahi*.

Coliforms are the indicators of fecal or sewage contamination (Cominazzini, C. 1978). The presence of coliforms indicates that many *Dahi* of Kathmandu have fecal contamination and have poor sanitary condition. All four coliform were isolated from the *Dahi* samples. Most common coliform was *E. coli* which was detected in 96.87% (31/32) coliform positive samples. It is followed by *Klebsiella pneumoniae* 46.87% (15/32). Each *Citrobacter frundii* and *Enterobacter aerogenes* are present in only 6.25% (2/32) of coliform positive samples. Coliform are not only the indicator organisms but also undesirable contaminants of *Dahi*. Their contaminations produce off flavor and

undesirable changes which reduced the quality of the product. Coliform like Citrobacter and Enterobacter produces bitterness, and acid or sour flavor. *E. coli* and *Klebsiella spp* causes excessive gas production, foaming and ropiness (Frazier and Westhoff, 2001). Only 54.92% (39/71) samples meet the DDC standard (less than 10 cfu / ml) criteria for coliform.

Indicator organisms are used in safety and quality control procedures where potential problem organism can't be detected with ease and reliability. For safety, an indicator organism should be at least resistant or persistent as the problem organism. (Atlas and Bartha, 2005). There probably is no universal indicator for determination of microbiological quality of food and water. Under different conditions, different populations may be better than others (Wolf 1979). The detection of actual entero-pathogen such as *Salmonella* and *Shigella* in routine monitoring studies would be difficult and uncertain undertaking. The most frequently used indicator organism for fecal contamination is the normally nonpathogenic *E. coli* (Atlas and Bartha, 2005).

In this research 92.95% (66/71) samples were found to be positive for Staphylococci. The highest and the lowest count were 4.69×10^5 and 33 Cfu / ml respectively with mean 3.18×10^4 Cfu / ml. The Staphylococcal load was in the range of 10^4 - 10^5 Cfu / ml in 46.47% samples. This is in agreement with result where the mean *Staphylococcus* count for fermented milk (fura) and butter were 4.3 x 10^4 cfu / ml and 2.2 x 10^4cfu / ml respectively (Umoh, 1990) 31. In a study done in home made yoghurt in Karak of Jorden the mean Staphylococci load was found to be 4×10^3 cfu / ml (Riadh, 2005) which had lower load than our study.

In Nepal there is no guideline for Staphylococcal load in *Dahi* but in comparisons to the ICMSF guideline (1997) 52.11 % (37/71) did not meet the standard limit. It means that most of *Dahi* have poor quality and are not safe for consumption. Consumption of *Dahi* of Kathmandu has potential of Staphylococcal intoxication.

Bacillus cereus was the second most contaminated among studied organisms in *Dahi* after yeasts. It was found that 88.73% of samples were positive for *Bacillus cereus,* the highest and the lowest count was 2.99×10^6 and 4×10^3 Cfu / ml respectively with mean 5.51×10^5 Cfu / ml. Among 71; 36 samples (50.71%) were in the range 10^5-10^6 cfu / ml. In a research it was found that 100% raw and 26% sterilized and 86% pasteurized milk sample were contaminated with *Bacillus cereus* (Dabadi, 2008). According to Notermans *et al.* (1997) the yoghurt of The Netherlands contains 10^5 to 10^6 *B. cereus* ml^{-1}. It is common saprophytic environment microorganism and it's contamination in various food is common. It can form spore and can withstand the commercial pasteurization temperature. Its post pasteurization contamination during storage and handling also adds for its greater load in dairy products. *Bacillus cereus,* a normal contaminant of milk, can be expected to be present in low numbers. Its mere presence does not constitute a hazard, but if the product is sufficiently abused to permit growth to high numbers, a significant hazard could result. (ICMSF, 1986) *Dahi* is prepared from the milk for which additional handling is essential. Additional handling during pouring of milk into *Dahi* vessels increase the exposure to the environment which accounts for more load of environmental organisms like *Bacillus* ***cereus***. Seeding or addition of starter to the milk also increases the load. If the milk is previously contaminated with *Bacillus cereus* and addition of seed contaminated with the organism drastically increase the count. The count of *Bacillus cereus* in ceramic cups is higher than that of plastic cup and open *Dahi* indicates that ceramic cups itself adds the organism. During sample collection survey it was found that ceramic cups are prepared and stored in dusty rooms for several months. The same cups are just washed with tap water and used to make *Dahi*. It will be the main reason for increased number of *Bacillus cereus* in ceramic cups. Plastic cups *Dahi* samples have slightly greater number of Bacillus cereus than of open *Dahi* it will be due to more handling and contamination of plastic cups.

Almost all samples 98.60% (70/71) were found to contain fungi. The mean Yeast and mold count was found to be 2.5×10^7 cfu / ml. The highest count was 1.07×10^8 cfu / ml and the lowest was 5.16×10^5 cfu / ml among the Yeast and mold positive samples. The

common range for yeasts and mold is 10^7 to 10^8 cfu/ ml where 73.24 % of observation occurred. The yeast and mold load in *Dahi* varies from 10^5 to 10^8 which are shown in the table. This finding is in agreement with the study done by NDDB in 2001in which 98.04% samples contained yeasts and molds with the mean value 3.4×10^7 cfu / ml. The yeasts and mould count varied from 10^3cfu / ml to 10^{10} cfu / ml and the common range for yeast and moulds was 10^6 to 10^7 cfu / ml. (Waldhauer *et al*., 2001). Similar study revealed that moulds and yeast were present in 80 % and 50 % of raw milk and yoghurt samples respectively, with mean values of $4.3\times10^5 \pm 2.5\times10^5$ and $2.1\times10^4\pm1.9\times104$ cfu / ml, respectively. *Aspergillus* spp., *Cladosporium* spp., *Mucor* spp., *Curvularia* spp., *Penicillium* spp., *Geotricum* spp., *Candida* spp., *Rhodotorula spp., Torulopsis* spp. *and Saccharomyces* spp. isolated from both raw milk and yoghurt (El-Diasty and El-Kaseh, 2007).

Most of yeasts are harmless and do not cause any disease. Yeasts utilize the lactose of milk and also produce acid for curd formation. However excess of yeasts in place of starter of *Dahi* have bad effects. The role of contaminating yeasts in reduction of self life of *Dahi* and in the spoilage has been investigated (Sukumar, 2002). It may interfere with the taste, flavor, texture, aroma and curdling time. Excess of yeasts in *Dahi* produce yeasty flavor which is not liked by many consumers also produce gases and foaming in the vat.

Aspergillus, Penicillium and Fusarium species are the most commonly occurring moulds in dairy products (Suman, 2000). Curd is the favourable medium for the growth of *Aspergillus* and *Penicillium* species. They will grow and produce mycotoxin "(Ochratoxins) if the temperature of storage is between 0ºC and 25ºC and cause health hazards to consumers (Kumaresan, 1994).

The diversity of moulds and yeast count in this study is similar to finding of Saravanakumar *et al.* (2007). According to them *Penicillium* spp., *Aspergillus* spp. were present in 43.4%, 36.7% of samples respectively and common mold were *Fusarium* spp., *Cladosporium* spp., *Mucur* spp., *Rhizopus* spp. Out of 10 *Aspergillus ochraceus*

isolates obtained from curd, only four isolates were able to produce ochratoxin in the artificial medium. The yeasts load were 1.92 x 10^6, 1.58 x 10^5 and 1.15 x 10^5 cfu/m for *Dahi* of bulk vendors, hotels and households respectively (Saravanakumar *et al.,* 2007).

Establishment and application of microbiological criteria for foods at any point in the food chain from primary production to final consumption and it implementation of criteria provides food safety and consumers health protection.

The safety of foods is principally assured by control at the source, product design and process control, and the application of Good Hygienic Practices during production, processing (including labelling), handling, distribution, storage, sale, preparation and use, in conjunction with the application of the HACCP system. This preventive approach offers more control than microbiological testing because the effectiveness of microbiological examination to assess the safety of foods is limited.

Microbiological criteria should be established according to these principles and be based on scientific analysis and advice, and, where sufficient data are available, a risk analysis appropriate to the foodstuff and its use. They should be developed in a transparent fashion and meet the requirements of fair trade. They should be reviewed periodically for relevance with respect to emerging pathogens, changing technologies, and new understandings of science (ICMSF, 1997).

The establishment of meaningful and logic microbiological criteria is a complex process, which is, however, not always performed correctly. Microbiological criteria define the acceptability of a product based on the presence/absence or number of microorganisms (and/or their toxins) per unit(s) of mass, volume, area or lot. Ideally they should be based upon a Food Safety Objective (FSO), i.e. a statement of the maximum frequency and/or concentration of microbiological hazards in a food (Cordier, 2004).

For microbiological quality safety, specification and assessment detection, and enumeration of various bacteria, yeasts, molds, algae, parasitic protozoa and helminthes

as well as their toxins/ metabolites is done. The microorganisms included in a criterion should be widely accepted as relevant, as pathogens, as indicator organisms or as spoilage organisms to the particular food. Organisms whose significance in the specified food is doubtful should not be included in a criterion.For example *Clostridium perfringens, Staphylococcus aureus* and *Vibrio parahaemolyticus* does not necessarily indicate a threat to public health.

Where pathogens can be detected directly and reliably, consideration should be given to testing for them in preference to testing for indicator organisms. If a test for an indicator organism is applied, there should be a clear statement whether the test is used to indicate unsatisfactory hygienic practices or a health hazard (ICMSF, 1997).

6.2 CONCLUSION

Microbiological examination of *dahi*, a prominent dairy product of Kathmandu was carried out by enumeration, isolation and identification of starter, indicator, pathogens and spoilage organisms. Only 4.23% of samples contained *Streptococcus thermophilus*, coliforms were detected in 54.92% of samples, 92.95% of samples contained *Staphylococci*, 88.73% samples contained *Bacillus cereus*, 98.60% of samples were found to be contaminated with yeasts and molds and 1.4% of samples were positive for *Salmonella* spp. None of the sample meets the standard prescribed by DDC, DFTQC, PFA, FAO and ICMSF. The result revealed that most of samples were found to be contaminated with those organisms thereby indicating very poor quality. Hence in order to maintain overall quality of *dahi* good hygienic practice and good manufacturing practice should be implemented for the prevention of public health hazards.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

1. The study was carried out at central department of microbiology Kritipur Kathmandu Nepal since July 2007 to Jan 2008 for six months.

2. Altogether 11 sampling sites, Baneshwor, Koteshwor Maharajgunj, Patan, Sanothimi, Bhaktapur, Kalanki, Krtitipur, Gangabu, Lainchaur, Ratna park area, and were selected for sample collection.

3. The study was designed to assess the storage and selling practices of *Dahi* being practiced by the sellers as well as microbiological quality especially to detect the bacteria of family Enterobacteriaceae, *Streptococcus thermophilus*,Staphylococci, *Bacillus cereus*, yeast and moulds in the *Dahi* of various industries of Kathmandu.

4. Different media and technique were used for study e.g., the Coliforms count in VRBA Yeast and mould count in PDA which is made selective by adding chloramphenical *Staphylococci* count in MSA, *Bacillus cereus* detection in *Bacillus cereus* selective media and *S. thermophilus* in M17.

5. Fifty five shelves, 35 refrigerated and 10 floors stored *Dahi* were taken as samples in which 34 plastic cups, 19 open (plastic bag) and 18 ceramic cups *Dahi* were included.
6. *S. thermophilus* was isolated from very few samples (4.27%) with mean count of 2.57×10^5cfu/ml and were found to be urease deficient.

7. In 45.08% (32/71) samples coliform were detected and all coliform positive samples have greater than 10cfu/ml. Among coliform, *E. coli* 96.87% (31/32)

Klebsiella pneumoniae 46.87% (15/32), and each *Citrobacter freundii and Enterobacter aerogenes* were detected in 6.25% of samples.

8. Staphylococci were detected in 92.95% (66/71) in which 87.87% (58/66) samples revealed coagulase positive staphylococci and 52.11 % (37/71) samples did not meet the standard of ICMSF.

9. *Bacillus cereus* was found in 88.73% of samples, the highest and the lowest count was 2.99×10^6 and 4×10^3 CFU/ml respectively with mean 5.51×10^5 CFU/ml.

10. Yeast and mold were detected in 96.6% (70/71) of samples with mean count 2.5×10^7cful/ml and were the most abundant among the studied organisms. *Geotricum* spp. 49.20% (31/61), *Aspergillus* spp. 44.44%, *Mucor* spp. 42.85%, *Penicillium* spp. 31.74%, *Cladosporium* spp. 7.93%, *Fusarium* spp. 9.52%, and *Rhizopus* spp. 6.34%, was present.

11. *Salmonella typhurium* was present in only one sample (1.45%) but not *Shigella* spp. in any.

7.2. RECOMMENDATION

The study about *Dahi* indicated the very poor microbiological quality and thus the food safety. This could have a major health hazards to the public health. The quality improvement process is a continuous process and it follows the plan "Do Study Act problems solving Cycle" It ever seeks for better quality. Based on this philosophy as well as the finding about the present status of the *Dahi* mentioned above, the following recommendations are made

1. In the present contest, quality issues are not properly mentioned or addressed because there is no proper comprehensive quality policy for milk and milk products at the nation level. This policy must be based upon quality assurance system than quality control system. Companies producing high quality of products should be appreciated and with low quality should be penalized.

2. Defined Starter of *Dahi* without contamination is highly recommended as most of samples didn't contain principal starter *Streptococcus thermophilus*.

3. Use of potable water, clean and sterile containers, high quality of raw materials i.e. milk and, *Dahi* additives and appropriate starters is highly recommended as they play important role in quality of *Dahi*.

4. National standard limits for various types of indicator as well as food borne pathogens should be developed for dairy products. If not so standard limits for common organisms should be developed or the reference standard of codex should be implemented.

5. There should be effective and lucid quality control program on the dairy products, which should ensure enforcement and regulation of microbiological standards.

6. Earth wares used as vessel in preparation of *Dahi* should be properly sanitized before use and hand touching and contamination of plastics bags or cups during selling should be avoided.

7. HACCP and its prerequisite should be implemented on milk chain.

8. *Dahi* should be stored, retailed, in an unbroken cooling chain after heat treatment in order to keep bacterial growth as low as possible and ensure as long as possible shelf life *Dahi*.

CHAPTER VIII

8. REFERENCES

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APPENDIX-I

Questionaire

Sample no.

Ward…… House block No…… Tole ……. District……

Business Name (Dairy industries)……

1. How long are you in this business? …….
2. What kind of business activities related to dairy are you engaged in?

A) Producer	Yes	No
B) Retailer	Yes	No
C) Wholesaler	Yes	No

3. What is your daily dahi production in liters? ……
4. Do you know that disease can be transmitted from dairy products?

	Yes	No

5. What sorts of Dahi do you produce?

A) Open *dahi*	Yes	No
B) Ceramic cup *Dahi*	Yes	No
C) Plastic cup *Dahi*	Yes	No

6. What type of seed (starter) do you use?

Own	Yes	No
Taken from other Dairy	Yes	No

Taken from other countries Yes No

7. Where do you store *Dahi?*

Shelves Yes No

Refrigeration Yes No

On Floor Yes No

8. Do you know about?

GMP Yes N o

GHP Y es No

APPENDIX-II

Sampling areas

Major location	Sampling area	No. of samples
Baneshwor	New- Baneshwor	6
	Mid-Baneshwor	2
	Thapagaun	1
	Sankhamul	5
	Buddhanagar	1
Koteshwor	Mahadevsthan	3
	Koteshwor Chowk	2
Maharajgunj	Maharajgunj Chowk	3
	Galfutar	1
	Lazimpat	2
	Panipokhari	1
Kalanki	Khashi bazzar	1
	Kalanki Chowk	1
Kirtipur	Naya bazzar	2
	Tyangla	1
Gangabu	New Buspark	1
	Ganeshthan	3
Lainchaur	Lainchaur	1
Ratnapark	Bagbazar	3
	Kantipath	1
	Mahabauddha	1
	New road	1

	Putalisadak	1
	Tapathali	1
Sanothimi	Sanothimi	1
Bhaktapur	Darbar Square	8
	Chorcha	1
	Gathaghar	1
	Ichharte	1
	Sallaghari	1
	Shrijana nagar	1
	Sukuldhoka	3
	Suryabinayak	2
	Talakhun	1
Patan	Kupandol	1
	Lagankhel	1
	Pulchowk	1
TOTAL		71

APPENDIX –III

List of Equipments and Materials Used During the Study

A. EQUIPMENTS

Hot air oven	Memmert (Japan)
Incubator	Sakura (Japan)
Autoclave	Water Distillation Plant

Refrigerator	Sanyo (Japan)
Microscope	Olympus (Japan)
Weighing Machine	Scaltec instruments (Germany)

B. MICROBIOLOGICAL MEDIA

Mueller Hinton broth	Bacillus cereus selective medium
Simmons Citrate agar	MacConkey agar
Sulphur Indole Motility agar	Nutrient broth
Mueller-Hinton agar	Triple Sugar Iron agar
Mannitol Salt agar	MR-VP medium Urea broth
Violet Red Bile agar	Urease agar
Salmonella-Shigella agar	Nitrate broth
Potato Dextrose agar	Nutrient agar

Polymyxin pyruvate egg-yolk mannitol bromothymol blue agar (PEMBA)

M17

C. CHEMICALS AND REAGENTS

3% Hydrogen peroxide	bromothymol blue
Crystal violet	Kovac's reagent
Gram's iodine	phenol red
Absolute (95%) alcohol	Safranine
α-naphthylamine	Sodium pyruvate

D. MISCELLANEOUS

Conical flasks, Cotton, Distilled water, Droppers, Forceps, Glass slides and cover slips, Immersion oil, Eggs, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder, Petri dishes, Pipettes, Plastic containers, Spatula, Test tubes, Wooden applicator sticks

APPENDIX-IV

I. Composition and Preparation of Different Culture Media

The culture media used were from Hi-Media Laboratories Pvt. Limited, Bombay, India. (All compositions are given in grams per liter and at 25^0C temperature)

1. MacConkey agar (MA)

(Without sodium taurocholate, without salt and crystal violet)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0
Neutral Red	0.04
Agar	20.0
Final pH (at 25^0C)	7.4±0.2

55 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

2. Mueller Hinton agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Acid Hyrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C)	7.4±0.2

38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve. 10 ml was distributed in test tubes and sterilized by boiling in water bath for 10 minutes.

3. Nutrient agar (NA)

Ingredients	gm/litre
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25^0C)	7.4±0.2

37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

4. Nutrient broth (NB)

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH (at 25^0C)	7.4±0.2

13 grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121^0C for 15 minutes.

5. Mannitol salt agar

Ingredients	gm/litre
Beef extract	1.0
Protease peptone	10.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH (at 250C)	7.4±0.2

111 grams was suspended in 1000ml distilled water, boiled to ensure complete solution and then sterilized at 15 lbs pressure (1210C) for 15 minute

6. Salmonella-Shigella(S-S) Agar

Ingredients	gm/litre
Beef extract	5.0
Peptone	5.0
Lactose	10.0
Bile salts	8.5

Sodium citrate	10.0
Sodium thiosulphate	8.5
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	15.0
Final pH	7.0±0.2

63 grams was suspended in 1000ml distilled water in sterilized conical flask and boiled with frequent agitation to ensure complete solution. It should not be autoclaved. It was cooled to 500C and poured.

7. Violet red bile agar

Ingredients	gm/litre
Yeast extract	3.0
Peptone	7.0
Bile salt no.3	1.5
Lactose	10.0
Sodium chloride	5.0
Agar	15.0
Neutral red	0.03
Crystal violet	0.002
Final pH (at 250C)	7.4±0.2

41.5 grams of the medium was suspended in 1000ml sterile distilled water in sterilized conical flask, heated to boiling to ensure the complete solution and poured into plate at 450C

8. Potato dextrose agar (PDA)

Ingredients	gm/litre
Potato infusion	200.0g
Dextrose	20.0g
Agar	15.0g
Distilled water	1000.0ml
Chloramphenicol	30mcg/ml
Final pH	5.6±0.2

39 grams of the medium was suspended in 1000ml sterile distilled water, heated to boiling to ensure the complete solution and autoclaved. Then it was cooled to 45C and poured into plate.

9. Bacillus cereus selective media (BCSM)

Ingredients	gm/litre
Agar	15.0
Sodium Pyruvate	10.0

Mannitol	10.0
Na2HPO4	2.5
NaCl	2.0
Peptone	1.0
KH2PO4	0.25
Bromothymol blue	0.12
MgSO47H2O	0.1
Egg yolk emulsion	25ml

Preparation of egg yolk emulsion
Eggs were soaked with 1:100 dilution of mercuricchloride solution for 1 minute. Eggs were cracked and separated the yolk from the white. Twenty milliliter (20ml) of egg yolk was measured and mixed with 80ml of 0.9% NaCl solution. It was mixed by shaking and warmed to 45C.

10. Polymyxin pyruvate egg-yolk mannitol bromothymol blue agar (PEMBA)

Ingredients	gm/litre
Peptone	1
Sodium pyruvate	10
D-mannitol	10
$MgSO_4.7H_2O$	0.1
NaCl	2
Na_2HPO_4	2.5
KH_2PO_4	0.25
Bromothymol blue (water soluble)	0.12
Polymyxine B solution	10.0ml
Agar	18
Egg-yolk emulsion	5ml
pH	7.2

Preparation of egg yolk emulsion
Eggs were soaked with 1:100 dilution of mercuricchloride solution for 1 minute. Eggs were cracked and separated the yolk from the white. Twenty milliliter (20ml) of egg yolk was measured and mixed with 80ml of 0.9% NaCl solution. It was mixed by shaking and warmed to 45^0C.

11. M17

Ingredients	gm/litre
Caesin enzymic hydrolysate	2.5
Peptic digest of animal tissue	2.5
Papaic digest of soya bin meal	5
Beef extract	5
Yeast extract	2.5
Ascorbic acid	0.05

Magnesium Sulphate	0.25
Lactose	5
Disodium glycerophosphate	19
Final pH	7.1±0.2

42.25 gram of media is suspended in 1000 ml of distilled water and, heated to boiling to ensure the complete solution or can be autoclaved. Then it was cooled to 45^0C and poured in to plates.

II. **Biochemical Test Media**

1. MR-VP Medium

Ingredients	gm/litre
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^0C)	6.9±0.2

17 grams was dissolved in 1000 ml distilled water. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes.

2. Hugh and Leifson's Medium

Ingredients	gm/litre
Tryptone	2.0
Sodium Chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0
Final pH (at 25^0C)	6.8±0.2

9.4 grams of the medium was rehydrated in 1000 ml cold distilled water and then heated to boiling to dissolve completely. The medium was distributed in 100 ml amounts and sterilized in the autoclave for 15 minutes at 15 lbs pressure (121^0C). To 100 ml sterile medium aseptically added 10ml of sterile Dextrose and mixed thoroughly and dispensed in 5 ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium

Ingredients	gm/litre
Beef Extract	3.0
Peptone	30.0

Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH (at 25^0C)	7.3±0.2

36 grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized.

4. Simmon Citrate Agar

Ingredients	gm/litre
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25^0C)	6.8±0.2

24.2 grams of the medium was dissolved in 1000ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

5. Triple Sugar Iron (TSI) Agar

Ingredients	gm/litre
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25^0C)	7.4±0.2

65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

6. Christensen Urea Agar

Ingredients	gm/litre
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

24 grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at 121^0C for 15 minutes. After cooling to about 45^0C, 50 ml of 40% urea was added and mixed well. Then 5 ml was dispensed in test tube and set at slant position.

7. Nitrate broth

Ingredients	gm/litre
Potassium nitrate	0.2
Peptone	5
Distilled water	1000ml

Five milliliter (5ml) was added in tubes and autoclaved at 121^0C for 15 min.

III. **Staining and Test Reagents**

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W) to make 1 litre	

Preparation: In a clean piece of 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 was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally the volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Preparation: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Preparation: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Preparation: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

3. Normal saline

Sodium Cholride	0.85 g
Distilled Water	100 ml

Preparation: The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

4. Test Reagents

a. For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

b. For Oxidase Test

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

c. For Indole Test

Kovac's Indole Reagent

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Hydrochloric acid	10 ml

Preparation: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

d. For Methyl Red Test

Methyl Red Solution

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Preparation: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

e. For Voges-Proskauer Test (Barritt's Reagent)

Solution A

α-Napthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Preparation: To 25 ml D/W, 5 g of *α*-Napthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Preparation: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

f. Solution A for Nitrate reduction test

Sulphanilic acid	8g
Acetic acid (5M)	1litre

Preparation: To 100 ml acetic acid (5M), 8 g of sulphanilic acid was dissolved and transferred into a clean brown bottle. Then the final volume was made 1000 ml by adding acetic acid (5M).

g. Solution B for Nitrate reduction test

α-naphthylmine	5g
Acetic acid (5M)	1litre

Preparation: To 100 ml acetic acid (5M), 8 g of α-naphthylmine was dissolved and transferred into a clean brown bottle. Then the final volume was made 1000 ml by adding acetic acid (5M).

APPENDIX-V

A. Gram-staining Procedure

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

APPENDIX-VI

1. Biochemical Tests for Identification of Bacteria

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.During aerobic respiration, in the presence of oxygen, microorganisms produces hydrogen peroxide, which is lethal to the cell itself. 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% H_2O_2 was put on the surface of the 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 (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria, Alcaligenes, Aeromonas, Vibrio, Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24

hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

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 color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C 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 color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges- proskauer- negative or methyl red negative and Voges-Proskauer positive. The Voges proskauer test for acetoin is used primarily to separate *E. coli* from *Klebsiella* and *Enterobacter* species

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt'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 color.

F. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon’s Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C 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. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility test

This test is 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 a single flagella. The motility media used for motility test are semisolid, 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 the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/ Sucrose fermenter, H_2S producer.

b. Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production.

c. Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.

d. Yellow (Acid)/ No Change → Glucose- Oxidiser.

e. No Change / No Change →Non-fermenter.

I. Urea Hydrolysis test:

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) 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°C overnight. Positive organism shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

J. Coagulase test

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. saprophyticus, S epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *S. aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

Slide Coagulase Test

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. 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: For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

Tube Coagulase Test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin

which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

Procedure: In the tube coagulase test, plasma was diluted 1:10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*S. aureus* culture), and 0.5 ml negative control (*S. epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37^0C on a water bath for 6 hours and observed for gel formation in every 30 minutes. The clotting is observed by gently tilting the tube for positive coagulase test.

K. Nitrate reduction test

This is a test for the presence of the enzyme ntrate reductase which causes the reduction of nitrate, in the presenc of a suitable electron donor, to nitrate which can be tested for by an apporpirate colorimetric reagent. Almost all Enterobacteriaceae reduce nitrate.

Proceudre: Nitrate medium was inoculated with test organisms and incubated for 24h.Few drops of test reagents (Solution A and Solution B) were added to test culture.A red color developing within a few minutes indicates the presence of nitrite and hence the ability of the organisms to reduce nitrate.

APPENDIX-VII

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
C. freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E. aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
P.mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M. morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside.

Some strains of Serratia marcescens may produce a red pigment

Yersinia are motile at 22°C.{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}(Source: Collee *et al.* 1996)**.**

APPENDIX-VIII

1. Analysis of variance (ANOVA) for microbial count according to type of containers**.**

Type of container	Coliform (X_1)	Yeast (X_2)	Staphylococci (X_3)	Bacillus cereus (X_4)	Molds (X_5)	Sum
Plastic cup (Y_1)	1.38×10^4	2.7×10^7	7.63×10^4	5.62×10^5	3.61×10^3	$\sum Y_1=2.76\times10^7$
Ceramic cup (Y_2)	2.18×10^4	2.2×10^7	4.38×10^4	7.29×10^5	2.73×10^3	$\sum Y_2=2.27\times10^7$
Open (Y_3)	9.94×10^3	2.5×10^7	1.11×10^4	3.61×10^5	4.23×10^3	$\sum Y_3=2.53\times10^7$
Sum	$\sum X_1=4.55\times10^4$	$\sum X_2=7.4\times10^7$	$\sum X_3=1.31\times10^5$	$\sum X_4=1.65\times10^6$	$\sum X_5=1.05\times10^4$	

H_0: There is no difference of microbial count between types of containers and categorized microbial counts
H_1: There is difference of microbial count between types of containers and categorized microbial counts

$\sum X_1^2+\sum X_2^2+\sum X_3^2+\sum X_4^2+\sum X_5^2 = 6.08\times10^{14}$

$T=\sum X_1+\sum X_2+\sum X_3+\sum X_4+\sum X_5 =7.58\times10^7$

$T^2=5.74\times10^{15}$

$T^2/N=5.74\times10^{15}/15$
$=3.83\times10^{14}$

$TSS=\sum X_1^2+\sum X_2^2+\sum X_3^2+\sum X_4^2+\sum X_5^2-T^2/N$
$=6.08\times10^{14}-3.83\times10^{14}$
$=2.25\times10^{14}$

$SST=(\sum Y_1)^2/n_1+(\sum Y_2)^2/n_2+(\sum Y_3)^2/n_3-T^2/N$
$=0.01\times10^{14}$

$SSM=(\sum X_1)^2/n_1+(\sum X_2)^2/n_2+(\sum X_3)^2/n_3+(\sum X_4)^2/n_4+(\sum X_5)^2/n_5-T^2/N$
$=-1.80\times10^{14}$

SSE=TSS-SST-SSM
$=4.04\times10^{14}$
ANOVA TABLE

Sources of variation	SS	d.f.	MS	F	F at 1%	F at 5%
Due to container	0.01×10^{14}	2	0.5×10^{12}	0.01	10.92	5.14
Due to count	-1.8×10^{14}	4	0.45×10^{14}	0.9		
Error	4.04×10^{14}	8	0.5×10^{14}			
Total	5.85×10^{14}	14				

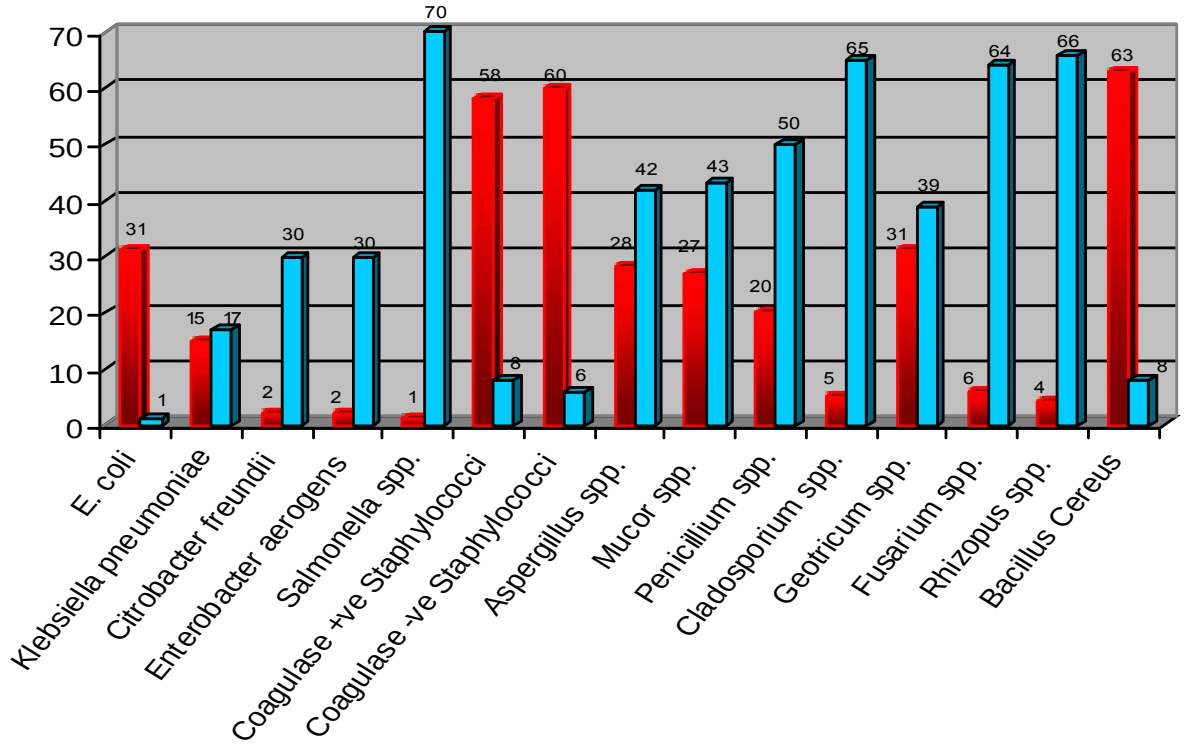

Figure 3: Pattern of microbial isolates from Dahi / yoghurt