A STUDY OF NAK CHEESE: FUNCTIONAL ASPECTS

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This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. We, hereby, recommend this thesis for final evaluation.

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ABSTRACT

Previous studies on functional aspects of *nak* cheese are scarce. This research work aims to study the functional aspects of *nak* cheese produced in Nepal with respect to its probiotic potential, and presence of phytochemicals. Three *nak* and a cow cheeses were collected randomly from regional factories of Dairy Development Corporation (DDC) in Nepal, located at altitude of 2900 m, 2600 m, 2400 m and 1900 m, respectively. Total probiotic Lactic acid bacteria (LAB) present in these cheeses were enumerated on MRS media with 0.25 % bile salts. The total viable probiotic bacteria in the studied cheeses were lower than bacterial 10^6 cfu/g). In this study, *nak* cheeses resulted higher probiotic count than cow cheese. Ripening duration of the cheeses was found to be an important factor affecting the viability of LAB. Total of 32 probiotic isolates obtained from the cheeses were studied for their suitability as gastro-intestinal probiotics. Only 12 isolates were able to tolerate pH 3.0 for 3 h, 0.25% bile salts for 4 h and show antibacterial activity against the *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively. These potential probiotic isolates were presumptively identified as *Streptococcus thermophilus* based on morphological observation and biochemical tests. Three *nak* cheese isolates, 2 cow cheese isolates and a commercial probiotic strain (*Bifidobacterium animalis*) were respectively used to prepare 3 yoghurt types under identical laboratory conditions. The stability of yoghurts prepared from cheese isolates was higher than yoghurt prepared from the commercial strain whereas viability of commercial strains was higher than cheese isolates in the yoghurts during 7 days of refrigeration. All the 3 yoghurts received similar preferences by the untrained panelist during sensory evaluation.

Three medicinal plants foraged by *nak* and the cheeses were subjected to phytochemical extraction using methanol as solvent followed by quantification of the phenol and flavonoid content in their extracts. The total phenol content (TPC) of the dry plant sample (DS), viz., Kag ko ankha (*Galtheria nummularioides)*, Sunpati (*Rhododendron lepidotum)* and Pati (*Artemisia vulgaris)* and for dry extract (DE) of cheeses *viz., nak* cheese(2900 m), *nak cheese* (2600 m), *nak* cheese *(*2400 m*)* and cow cheese (1900 m) were 67.30 mg GA/g, 112.02 mg GA/g, 91.72 mg GA/g, 10.9 mg GA/g, 9.79 mg GA/g, 6.06 mg GA/g and 2.75 mg GA/g respectively. Their corresponding total flavonoid content (TFC) were 23.88 mg QE/g, 79.27 mg QE/g, 39.66 mg QE/g, 7.70 mg QE/g, 5.45 mg QE/g, 4.12 mg QE/g and 1.80 mg QE/g respectively. The TPC and TFC of the medicinal plants were found as expected significantly higher ($p<0.05$) than the cheeses. *Nak* cheese (2900 m) contained a significantly higher ($p<0.05$) antioxidant activity (IC_{50}) of 371.64 mg/L among the studied cheeses. In contrast, cow cheese (1900 m) gave the lowest IC_{50} of 626.24 mg/L. The 4 cheeses extracts were also assayed for their effect on their respective probiotic isolates and toxicity towards brine shrimp. Cheeses extract exerted positive influence on the viability of probiotic isolates at 4 h of incubation at 37^{o}C. The *nak* cheese (2600 m) showed significantly higher ($p<0.05$) LC_{50} of 350.19 µg/mL (i.e., moderate toxicity) than other cheeses. Thus, *nak* cheese doesn't meet the recommended minimal population of 10^6-10^7 cfu/g for any probiotic strain of LABin a

product at the end of shelf-life (Miller, 2004) to have their functional aspects but it was found to have predominant presence of *Streptococcus thermophilus* species at its latter stage of maturation. It is concluded that *nak* cheese may be considered as good source of antioxidants and its regular consumption might improve consumer health due to antioxidant activity and biogenic effect of LAB.

Keywords: ***Nak* cheese, probiotics, phytochemicals, antioxidants**

CHAPTER I

INTRODUCTION

1.1 General introduction

1.1.1Nak Cheese

Female yak are called "*dri*" in Tibet; "*nak*" in Nepaland male is called yak. They are scientifically called*Bosgrunniens (*Linnaeus, 1766). Yak and nak reared at higher altitude above 3,000 m. Yak is raised in 22 Himalayan districts of Nepal along the Tibetan boarders with long hairs and pointed horns. The adult body weight of a male is about 245 kg and of female *nak* is about 215 kg. Yak and *nak* provides milk and meat for human consumption. *Na*k is very hardy animal and nomadic in nature. The mouth parts are adapted to graze very short grasses, quickly and efficiently. Their legs and hooves are very strong and suitable to walk in steep terrain. They can thrive on poor quality roughages. The *nak* remains in alpine pastures (3000-4500 m) for two months of July and August and the rest of the year in lower altitude oak forest (2500-3000 m)(Thapa, 1994). Unfortunately *nak* is unable to survive at lower altitudes and gives only 1-2 liters of milk in a day on average. Hybrid of nak called *nak*-cattle hybrid (Chauri) are produced by crossing Yak with female cow called Aulo and nak with local bull known as Kirko. The hybrid is thus able to survive at lower altitudes (upto 1800 m) and has high milk production (485 litres on average during 6 months of lactation). The hybrid milk has fat content about 5-6% whereas a pure *nak* has 7-9% milk fat (Indra , 1997).

Nak cheese also popularly known as "Yak cheese"is produced from *nak* or *nak*-cattle hybrid. *Nak* cheese made in Nepal has half the butterfat skimmed off the milk to make a harder-style cheese and is soaked in brine and cured outdoors in shelters. The cheese ends up grainy and tangy (Thapa, 1994). There is difference in feeding pattern between *nak* andother dairy cattle. Nak spends most of its time in pasture and are 100 % grass-fedcompared to dairy cattle which as mostly fed with grain-based diets. Therefore *nak* cheese have three times more beneficial ω-3 fatty acids, CLA and antioxidants than cow cheese even if the actual quantities are still small(McGee, 2008).

1. History of Nepalese nak cheese

Nepali cheese production started the same year as the first successful ascent of Everest. *Nak* cheese is the popular name for a cheese produced from *nak* and *nak* cattle-hybrid milk. These animals can exploit pastures from 6,000 to 18,000 ft. *Nak* cheese production began as a way to use surplus milk after the Tibetan market was closed to Nepalese due to the Chinese invasion (Dubach, 1990).Werner Schulthess, a swiss national dedicated a lot of his

time taking care of the production, distribution and marketing of the milk.Schulthess saw better prospects in the milk produced higher in the mountains and he thought of transporting it in the form of cheese to Kathmandu Valley. He worked hard with his Sherpa friends for several months to introduce cheese to the public for free. The locals loved the taste and this resulted in his relentless journey to start the cheese plant and factories(McGee, 2008).Establishment of cheese industry was assisted by the Food and Agriculture Organization and Swiss cheese makers.The first *nak* cheese factory was built in 1952 (Schulthess, 1986).Nepal became the first country in Asia to make any Western-style cheeses. Making of Swiss- style hard cheese was taught by the Food and Agriculture Organization of the United Nations (FAO) in 1952, so that Nepalese could earn cash incomes. Cheese production in Nepal remained in the sole hands of the Dairy Development Corporation (DDC). In the early 1990s, private cheese making became permitted. DDC-made *nak* Cheese is still considered safer and better (McGee, 2008). *Nak* cheese factories are in remote high hill areas of Nepal. It takes from 2 to 4 days to transport cheese from these factories to Kathmandu. The primary market of *nak* cheese is foreign tourists and ex-patriots living in Nepal (Colavito, 1994).There are altogether 20 *nak* cheese (11 under Dairy Development Corporation and 9 under private sector) (Pande, 1996).

2. Production of nak cheese

Nak cheese production is based on a Swiss recipe for a har*d* type *Guerre cheese a*dapted to Nepal's conditions. Major equipment required are a 300 liter copper pot, a milk separator, milk cans, cheese hoops, cheese cloth, brine tank, various tools and shelter. Cheese production also requires running clean cool water and two inputs that must be purchased from outside Nepal: starter culture and rennet. Cheese production also requires firewood to heat the cheese milk. The *nak* cheese recipe is designed for starter culture and rennet produced in Denmark (Colavito, 1994).The cheese factories in Nepal do not have their own Yak farm, so they collect milk from various farmers in the locality. The milk that qualifies the minimum criteria to make cheese is first poured into a milk can of 40 liters of capacity and it is then sink into water boiler for 30 minutes mentioning 65^{o}C temperature. The can is then drawn from the boiler and held into a cooler where it mentions 35^{o}C temperature. For the purpose of making cheese, milk should contain only 35% fat so by mixing skim milk with the fatty milk in a proportion thefat content is reduced to 35%. This mixture is then poured into a cheese kettle where its temperature reaches 33-35^{o}C(Pokhrel, 2012).

The mixed cheese culture consisting of *Lactobacillus helveticus* and *Streptococcus thermophilus*is added in skim milk to make mother culture. This mother culture is then poured in the cheese kettle in the proportion of 1:100, i.e. 1% of mother culture is mixed in the cheese kettle, and after 10 min *Renin* powder is mixed in the ratio of 2.5 gram per 100 liter of milk. The milk is then stirred and kept for 30 min to become perfectly yoghurt. Again the yoghurt is splited into 3X3X3 mm dimensions by cheese *herpes* which is again pressed for 30 min and finally the cheese is cooked at temperature of 50^{o}C for 30 minutes. The cooked cheese is tested either by chewing or by free hand. The cheese are now pressed in

order to make definite shape for 3 times in various interval of time called molding and finally the green cheese is ready, this green cheese is brought to brain tank (24 % salt) for blanching carried out for 48 h. This cheese is ripened for 3-4 months at 10-16^{o}C and is washed daily with salt water(Pokhrel, 2012).

1.1.2Some medicinal plants foraged by nak

Alpine pasture and Oak forest where *nak* forage are rich in plants of medicinal significance. Many of such plants are still unidentified. Some identified medicinal plants that are widely found in the pasture and consumed by *nak* as a part of their feed are mentioned below

A. ***Rhododendron lepidotum*** (family Ericaceae) is ahighly variable, low and compact to upright, lean shrub, up to 2 m tall white, yellow, pink, or purple colored flowers. The leaves are elliptic with a dark green upper surface and a lower surface with brownish scales. The distribution of *R. Lepidotum* is from Kashmir to southeast parts of Tibet at an altitude of 2400 to 4900 m. The genus Rhododendron has a long history of use as folk medicine in various countries. The leaf and flower powder of *R. lepidotum* have been used as a snuff in the treatment of headache (Lamichhane et al., 2010).

B. ***Artemisia vulgaris*** belongs to genus Artemisia and also known as mugwort. Mugwort have been used medicinal and as culinary herbs(Wikipedia, 2015). *Artemisia vulgaris* is a tall herbaceous perennial plant growing 1–2 m tall and has woody root with long and dark green colored leaves. The stems are erect and grooved with red-purplish tinge. The florets are radially symmetrical with many yellow or dark red petals. It flowers from July to September. It has stimulant and slightly tonic properties, and is of value as a nervine and emmenagogue, having also diuretic and diaphoretic action (Wikipedia, 2015).

C. ***Gaultheria nummularioides*** is a species of plant in the family Ericaceae. This species has been cultivated as an ornamental in Britain, the United States, and elsewhere for some decades.*Gaultheria nummularioides* is an evergreen Shrub growing to 0.1 m (0ft 4in) by 0.5 m (1ft 8in). It is in flower from Jul to August, and the seeds ripen from Dec to March. The flowers are hermaphrodite (have both male and female organs) and are pollinated by insects.The plant is self-fertile (Fang and Stevens, 2005).

1.2 Background

Modern lifestyles and longer life are also linked to various mental health problems such as depression, loss of memory chronic non-communicable diseases such as CVD, high blood pressure and type 2diabetes. In such concerns cheese with functional aspects can be a better solution.

As reported by McIntosh et al., (1995) the presence of phytochemicals in the milk and later in the cheese might be the result of their transfer from plant to milk. Similarly the secondary microflora of cheese consisting of yeast, LAB and mold are reported to produce phytochemicals such as phenols and flavonoids. These phytochemicals can influence milk and cheese taste and can also affect their antioxidant activity. Similarly, geographical location determines the content of phytochemicals (antioxidants) in plant (Taylor et al., 1997) and antioxidants peptides in raw milk of animals (Alyaqoubi et al., 2015). Ripening period of cheese (Yasudaet al., 2012) and its microbiology which consists of LAB, yeast and mold that can produce phenols and flavonoids (Sosa et al., 2001).Overwhelming evidence from epidemiological, in vivo, in vitro, and clinical trial data indicates phytochemicals can reduce the risk of chronic disease, particularly cancer (Block et al., 1992)

Fermented dairy product such as cheese could contain probiotic LAB present naturally through milk or through environment (Coppola et al., 1997). Qualities of cheese such as pH levels, low titrable acidity, high buffering capacity, more solid consistency, relatively higher fat content, high nutrient availability and low oxygen content protect probiotic LAB bacteria during storage (Karimi et al., 2011; Ong et al., 2006). There are variety of health benefits attributed to probiotics but their anti-carcinogenic, hypo-cholesterolemic and antagonistic actions against enteric pathogens and other intestinal organisms have been given the most attention (Mital and Garg, 1995). Researchers have found that cheese extract show higher cytotoxic effect in undifferentiated HL-60 cells than differentiated HL-60 cells at the varying concentrations tested (Yasuda et al., 2012).

Nepal was the first country in Asia to make any Western-style cheeses, and until the 1980s certainly the only Asian country making *Yak* Cheese. *Nak* cheese has become an important part of the regular diet of people at higher altitudes and being consumed by most of the Nepalese. *Nak* milk and cheese has become an important ingredient of diet for the people residing in higher altitudes to cope with oxidative stress brought about by direct UV exposure and rarefaction of the atmosphere and has helped them to stay healthy and live longer (Askew, 2002). Researcher have reported that *nak* cheese have three times more beneficial ω-3 fatty acids, CLA and antioxidants than cow cheese even if the actual quantities are still small(McGee, 2008).

Although some nutritive value of cheese has beenstudied, but yet many of the functional aspects of *nak* cheese manufactured in the hilly regions are to be evaluated. Nominal works have been carried in certain attributes of such products such as their probiotic potential and antioxidant value, which help to establish their functional potential in relation to fulfillment of their dietic and/ or therapeutic purposes as a result of which commercial value of *nak* cheese have been shadowed. Most of the cheese from cow, goat and camel's milk have been analyzed for the presence of phytochemicals, probiotics and their cytotoxicity beenevaluated, *nak* cheese still remains unexplored in many of such aspects.

1.3Statement of hypothesis

This research work was based on 3 main hypothesis which are listed below

1. *Nak*is acclimatized to cold climate of higher altitudes, forage on variety of flora and fauna of the Alpine Pasture and feed on rare and medicinally important herbs, have long extent of exposure to green vegetation and its secondary microflora consists of bacteria, yeast and molds. So the *nak* cheese could contain high content of phytochemicals as well as antioxidant proteins which might impart anti-oxidative effect to it.
2. *Nak* cheese is a fermented dairy product and could contain probiotic LAB present naturally through milk or through environment. Similarlystarter culture used for cheese production haveprobiotic potential.Nak cheese has higher pH levels, lower titrable acidity, higher buffering capacity, more solid consistency, relatively higher fat content, higher nutrient availability and lower oxygen content. These qualities protect probiotic LAB bacteria during storage. Therefore nak cheese could support adequate probiotic population and thus might have functional property conferred by probiotic LAB.
3. *Nak* cheese are ripened over 3 months andcould cytotoxic potential due to secondary microbial metabolites. Similarly, possible presence of phytochemicals and antioxidants could also result in its cytotoxicity.

1.4Objectives of the study

1.4.1 General objective

The general objective of the research work was to study the functional aspects of *nak* cheese obtained from high hills of Nepalwith respect to probiotic and phytochemical principles.

1.4.2 Specific objectives

The specific objectives of the research work are given below:

- To enumerate probiotic population in *nak* and cow cheese
- To screen the LAB capable of reaching the intestine in adequate population
- To prepare yoghurt from cow and nak cheese isolates and commercial probiotic strain for comparative organoleptic evaluation
- To carry out the phytochemical analysis of plantsforaged by*nak*
- To estimate phenol content, flavonoid content and antioxidant activityof *nak* and cow cheese
- To assay the influence ofdifferent cheese extracts on its respectiveLAB
- To evaluate the cytotoxicity of *nak* and cow cheese

1.5Rationale

Nepal was the first country in Asia to make any Western-style cheeses .The overall nutritive value of cheese is well established, but yet functional aspects*nak*cheese manufactured in the hilly regions are to be evaluated. Nominal works have been carried in certain attributes of such products that help to establish their functional potential in relation to fulfillment of their dietic and therapeutic purposes. *Nak* cheese still remains unexplored in many aspects such as the presence of phytochemicals, probiotics and their nutritional profile.

This study was conducted to comparatively assess the functional aspects of *nak* cheese produced in high hills of Nepal.Assessing the probiotic potential of *nak* cheese would establish its therapeutic significance considering the presence of viable bacterial count above suggested minimal of $>10^6$, else nak cheese could be considered as a source of probiotic bacteria which can be used in other short shelf-life products as starters.The study for the presence of phytochemicals and assay of antioxidant activity in *nak* cheese can be helpful in establishing its potential to treatment of oxidative stress as well as render its medicinal significance.Toxicity assay of *nak* cheese would evaluate its safety as well as anti-cancerous significance.The presence of antioxidantsalong with therapeutic significance of probiotic can enable*nak*cheese to be advocated as a healthier functional food.

1.6Scope

This research is a means to explore and promote internationally the functional potential of*nak* cheese. The presence of probioticsand phytochemicals addscommercial significance to these products. The phytochemicalspresent through milk due to consumption of medicinal plants by *nak*adds aesthetic attributes along with medicinal properties at zero risk. The product could contain medicinal value which can be complemented by therapeutic significance of probiotic and hence wide acceptance of theproducts. *Nak* cheese can be a potential source of probiotic bacteria that can be used in other dairy products such as yoghurt, ice-creams and probiotic drinks with short storage life and high viability of probiotic bacteria. The findings can enable nakcheese to compete with cheese from milk of cows and goats as well the ones that are imported. The findings can put this cheese to the preference list of Nepalese cheese lovers, which can directly or indirectly contribute to the health of the consumers, motivate the local cheese producers and also emphasize the need for preservation of endangered *nak*species. The outcomes of this research can also input positive perspective of national and international tourist towards these products and indirectly assist to tourism and economic development in the regions of their production. This research is an initiation and a minor attempt for understanding the potential *nak*cheese embraces. It is a nominal but significant exploration of this product, which is a delicious fermented dairy product of the higher altitudes.

1.7Limitations

*Nak*consume different flora and fauna at different season as well as have varied milk composition depending on time of the year so composition of the cheese is different at different season. The medicinal plants collected and cheese sampled are of a particular season and this research work does not represent overall scenario and the comparison cannot be made on seasonal basis. Similarly, probiotic potential of cheese can vary for various ripening stages owing todecreased viability of probiotics depending to somedegree on the sensitivity of the starters to salt on the water activity, and on theautolysis power of the strains at different length of product storage and conditions(Beresford and Williams, 2004). Therefore, single species of LAB were isolated from cheese. For a particular region of sample collection, cow and *nak* cheese could not be collected simultaneously,therefore the results of comparative analysis of different aspects of the study only applies to the selected cheese and cannot be forwarded to establish the overall superiority of a product to another. The study cannot confidently advocate *nak* cheese to be better than cow cheese but can favor nak cheese manufactured in any one region or altitude to be better than the other.

CHAPTER II

LITERATURE REVIEW

2.1 Functional aspects of cheese

As per the definition of Functional Food Center of the USA "Natural or processed foods that contain known or unknown biologically active compounds, in defined, effective, and non-toxic amounts, provided a clinically proven and documented health benefits for the prevention, management, or treatment of chronic diseases" are functional food (Martirosyan and Singh, 2015). These foods should improve state of health and reduce the risk of disease.

Functional aspects of food are related to foods ability to modulate one or more targeted functions relevant to a biological process or improved health based on accepted scientific research (Martirosyan and Singh, 2015). Example; Vitamin A is important for maintaining healthy eyes.Cheeseconsists complex mixture of specific bioactive proteins, lipids and saccharides. It contains numerous biologically active substances such as immunoglobulin, enzymes, antimicrobial peptides, oligosaccharides, hormones, cytokines and growth factors that might be present through milk (Pouliot and Gauthier, 2006). Mixture of antimicrobial agents that exhibit bacteriostatic and even bactericidal activities present in fresh milk can also be present in cheese. Milk proteins present in cheese are currently the main source of a range of biologically active peptides such as immune-peptides and lactoferrin. Cheese could potentially be used for improved health or wellbeing in a range of areas, including antimicrobial function, cardiovascular system, gastro-intestines, growth and metabolism, defense against free radical oxidation and to enhance psychological functions due to the presence of bioactive components such as antioxidants and probiotics (Abdel and A.M., 2010).

2.1.1 Phytochemicals

Phytochemicals are the compounds in plants (apart from vitamins, minerals, and macronutrients). Their effects include; antioxidant, boosting the immune system, and anti-inflammatory, antiviral, antibacterial, and cellular repair. Some of the well-known phytochemicals are carotenoids flavonoids, saponins and polyphenols. There are many phytochemicals and each works differently(Ganatraet al., *2012)*. Most phytochemicals have antioxidant activity. They protect our cells against oxidative damage, reduce the risk of developing cancer such as breast cancer, urinary tract infections and also improve dental health. Some isoflavones imitate human estrogens and help to reduce menopausal

symptoms and osteoporosis, Indoles, stimulate enzymes that make the estrogen less effective, Saponins interfere with the replication of cell DNA thus prevent the multiplication of cancer cells (Ganatraet al., 2012*).*

1. Flavonoids

Flavonoids are a group of polyphenols found mostly in plant flora and are used as antioxidants or free radical scavengers (Kar, 2007). They are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, flavones, dihydroflavons, flavans, flavonols, anthocyanidins proanthocyanidins, calchones and catechin and leucoanthocyanidins are some flavonoids found in plants (Kar, 2007).

2. Phenolic

Phenolic, phenols or polyphenols are chemical components that naturally occur as color pigments responsible for the color of fruits of plants. They play important role in plant defense against pathogens and herbivore predators and in control of human pathogenic infections (Puupponen*et al*, 2008). Phenolic are natural antioxidants, used as nutraceuticals, and found in apples, green-tea, and red-wine. They are even able to combat cancer and may even prevent heart ailments and inflammation (Kar, 2007).

2.1.2 Probiotics

Probiotics are defined as "Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host"(WHO, 2001). Microorganisms used as probiotics include Lactic acid bacteria (LAB), yeasts and molds.Health benefits of probiotics include, reduced incidence or severity of GI infections, alleviation of lactose intolerance and overall improvement in gut function including reduction in constipation as well as diarrhoea(Hoolihan, 2001).The criteria for a microorganism to be classified as probiotic are; human origin, nonpathogenic properties, resistant to technological processes, stable in acid and bile, adhesion to target epithelial tissue, able to persist within the GI tract, produce antimicrobial substances, able to modulate the immune system and able to influence metabolic activities (Szajewska, 2007).The major applications for probiotics are in dairy foods such as cheese (Wabel et al., 2008).

2.2Effect of altitude on total phenol and flavonoid content of forage plant

Plants are subjected to a wide spectrum of biotic and abiotic stresses. They try to cope with biotic pressures by synthesizing and allocating deterrents and toxicants (Rhoades, 1979), whereas for abiotic burdens plant alter their deposition of cuticular waxes, antifreeze polyols and aromatic compounds for selective sun radiation screening. Most of the

secondary metabolites have active chromophores that absorb UV radiation and others (flavonoids, tannins, alkaloids and phenolic) may also provide protection against climatic effects (Downum, 1992).

The tropical mountains receive highest levels of UV-A/B (280–380 nm) in the world due to atmospheric thickness and sun radiation angle and have steepest UV-B gradient in relation to altitude (Sullivan et al., 1992). Plant species differ in their ability to produce and accumulate phenolic materials which may be related to resistance to this selection force which may result their altitudinal segregation (Miguel et al., 2004). At higher altitudes there is decreased oxygen pressure,as a result reactive oxygen and nitrogen species (RONS) are formed at higher amount. These RONSare often may increase oxidative damage to lipids, proteins and DNA. Many researchers have reported that phenol and flavonoid synthesis is increased as preventive measures to protect cells and DNA against free radicals generated by such selective forces (Lois, 1994, Cuadra et al., 1997, Lalova, 1998, Markham et al., 1998 and Hofmann et al., 2000).

2.3Effect of feeding on milk quality and cheese

In recent years, several studies have shown that some plant compounds (e.g. vitamins,volatile organic compounds) are directly transferred from the grazed herbage to the milk (Fedele et al., 2000: Pizzoferrato et al., 2000). A large variety of the organic components present in the plant tissues can accumulate in the lipid and water-soluble fraction of the milk. Therefore the diet of the grazing animalscan affect the physico-chemical and organoleptic features of milk . The aroma of milk can be influenced by monoterpene compounds in particular. Similarly, terpenes could be used as biochemical indicators of the composition of the forage grazed and could be used to determine the origin of cheese (Fedele et al., 2000).

Researchers such as Dumont and Adda, 1978, Dumont et al., 1981 have studied the impact of animal diet on the monoterpenes and sesquiterpene content in cheese. In their study they have reported the frequent presence of these aromatic compounds in dairy products when animals are fed mountain forages. Similarly, Viallon et al. (1999), have reported that highland grass with different botanical composition strongly affects the flavour of milk and cheese. As reported by Connel and Fox, 2001, when the cows are fed large quantities of particular crops, the majority of phenolic compounds found in cow milk are derived from the feed. Other phenolic compounds may also be detected in ruminant milk. Moreover some alklphenols present in ruminant milk are derived from phenolics ingested through the animal feed (Kilic and Lindy, 2005).

Recent researchhas claimed that grass feeding improves the quality of the cheese and makes the cheese richer in ω-3 fats, vitamin E, and CLA. One ounce of grass-fed cheese can

provide at least 30 mg of CLA. Health benefits of CLA includessupport to immune and inflammatory system, improvement of bone mass, improvement of blood sugar regulation, reduction in body fat, reduction in the risk of heart attack, and maintenance of lean body mass. The amount of CLA in cheese tends to increase along with consumption of fresh grasses by the cows whose milk is used to make the cheese (Sacca et al., 2009).Additional benefits by grass-fed cheese are; increased levels of ω-3 fat, less palmitic acid and a better ratio of ω-6 to ω-3 fat. In recent studies, grass-fed cheese has been shown to provide a ratio of 4:1, at most; ratios of 3:1, 2:1, and even 1.5:1 are also often offered by grass-fed cheese (Sacca et al., 2009). Antioxidants found in 100% grass-fed whole milk can include the isoflavones formononetin, biochanin A, prunetin and lignans. Vitamin antioxidants include vitamin E is increased by about 50% in milk from 100% grass-fed cows than conventionally fed cows. Grass-feeding also increases the amount of ß-carotene in cow's milk. This level is about 4 times higher than in milk from conventionally fed cows (Coppa et al., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

Equipments provided by Central Department of Biotechnology, Kirtipur, such as centrifuge (Eppendorf AG 22331 Hamburg), sonicator (Indosati Scientific Lab equipments), hot air oven, Orbital shaking incubator (Ascent India), vacuum filter, rotavapour (IKA® HB 10 digital), spectrophotometer(GENESYS 10VIS) and hot air oven (Acumen Equipment India Pvt ltd) were used for the research.

3.1.2 Chemicals used

Sodium acetate, Methanol, DPPH, Folin–Ciocalteu reagent and ethanol manufactured by Thermo-Fisher Scientific India Pvt ltd, Potassium chloride, sodium carbonate, sodium hydroxide, calcium carbonate, Sodium Azide, Aluminum chloride manufactured by Merck specialties Pvt ltd, bile salts manufactured by Loba Chemie andGallic acid manufactured by Molychem were used in the research. Media such as MRS broth, MRS Agar, MHB, MHA, Nutrient agar, YGP broth,galactose and lactose manufacturedby HIMEDIA Laboratories Pvt Ltd were used for screening and isolation purpose.

3.1.3 Sampling of plants and cheeses

Four types of Commercial cheese including one cow cheese known as Kanchan cheese from Pashupatinagar, and three *nak* cheeses were sampled from cheese factories of Dairy Development Corporation. *Nak* cheeses were collected from Rasuwa, Jiri, Solukhumbu based DDC processing factories located at different altitudes (Fig 3.1).The samples were of varying ripening period (Table 3.1) with cow cheese ripened for a month and *nak* cheese ripened over three months.

Figure 3. 1: Cheese samples collected from regional cheese processing factories of Dairy Development Corporation.

Table 3. 1: Types of cheese sampled, altitude of sampling stations, ripening period of cheese in months and sampling date.

Cheese type	Location	Altitude(m)	Ripening period(m)	Sampling month
Cow	Pashupatinagar, Illam	1900	1	October
Nak	Parvatikunda, Rasuwa,	2400	4	November
Nak	Chordung, Jiri, Dolakha	2633	3	November
Nak	Bamti, Solukhumbu	2900	3	December

Three medicinal plant specimens foraged by *nak* were collected from Rasuwa(2400 mtrs) during the month of November and identified at species level in Central Department of Botany, Tribhuvan University, Kathmandu (Table 3.2)

Table 3. 2: Plants used as fodder for *nak*.

Local name	Scientific name	Altitude(m)
Sunpati	*Rhododendron lepidotum ssp. salignum*	2400
Kag ko Ankha	*Galtheria nummularioides ssp. D.Don var, elliptica*	2400
Pati	*Artemisia vulgaris*	2400

Cheese samples about 500 grams were collected in sterile plastic carriers from factories of DDC located at different regions of the country. The samples were transferred to the Lab at Central Department of Biotechnology, Kirtipur and stored in refrigerators at 4^{o}C for further use.

3.1.3 Media used

MRS agar (de Mans Rogosa and Sharpe) was used for isolation and subculture of LAB. MRS Agar is a medium for the cultivation and enumeration of *LAB*. It was originally developed in 1960 by de Man, Rogosa & Sharpe, the medium is suitable for most lactic acid bacteria and is intended as a substitute for Tomato Juice Agar.When acidified to pH 5.4 M.R.S. Agar can be used to enumerate *LAB.* Nutrition is provided by a mixture of carefully selected peptones, glucose, beef & yeast extracts whilst Tween® 80, magnesium and manganese sulphates act as growth stimulants. The composition of media is presented in appendix (Table 8.1).

3.2 METHODOLOGY

3.2.1 Microbiological analysis

1. Total LAB count and probiotic enumeration

The refrigerated cheese sampleswere subjected to the procedure for isolation. Pour plate technique was used to isolate theorganisms. Samplesweredirectly diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile sodium citrate (2% w/v) as described by Coeuret et al.,(2006).After incubation in MRS agar with 4 % calcium carbonate at 30^{o}C for 48 halso to take into account the mesophilic LAB with optimum growth temperature range of approximately 25°C to 31°C (Sohrabvandi et al., 2012),individual colonies were counted to determine the total LAB count. For total probiotic enumeration same procedure for total bacterial count was followed but MRS media was added with 0.25% bile salts and 0.05% cysteine for probiotic selection and incubated at 37^{o}C for 72 h with modification of the method described by Shah NP, (2000). Theselected colonies were purified with streak plate technique. Theisolates were examined according to their colony morphology, catalase reaction andGram reaction using standard protocol.

2.Selection of potential intestinal probiotics

For the selection of intestinal probiotics among the LAB obtained during enumeration, the major selection criteriachosen were, resistance to low pH, tolerance against bile salt and theantimicrobial activity.

a. Resistance to low pH

Resistance to pH 3 is often used in vitro assays to determine the resistance to StomachpH. Because the food stay in stomach for minimum 3 hrs, this time limit was taken intoaccount (Prasadet al., 1998)also pH 1 and 2 were considered for the assay .In order to screen the

Lactic acid bacteria for its resistance to low pH, 1ml of fresh enriched culture was inoculated in phosphate- buffered saline (PBS, pH 1, 2 and 3) and incubated at 37°C for 3 h (Collins et al., 1998) and observed for the viable colonies on MRS agar, on 48 hours of incubation at 37°C.

b. Tolerance against bile

Because the mean intestinal bile concentration is believed to be 0.3% (w/v) andthe staying time of food in small intestine is suggested to be 4 h (Prasad et al., 1998).The experiment was applied at this concentration of bile for 4 h. Nutrientbrothcontaining 0.3% and 0.25% bilewasinoculated with active cultures and incubated for 4 h.(Collins, et al., 1998) and observed for the viable colonies on MRS agar, on 48 hours of incubation at 37°C.Then the isolatesthat showed resistance to bile saltswere subjected to antimicrobial assessment.

c. Antimicrobial activity

The isolateswere tested for antagonistic activities against three bacteria pathogens (*E.coli, S. aureus and Pseudomonas aeruginosa*) (Hoseini, 2004). The pathogens were ATCC cultures provided by Central Department of Biotechnology, Tribhuvan University. The LAB strains were inoculated in nutrient broth(supplemented with 2% lactose to stimulate organic acid production) for 24 h at 37 ^{o}C and centrifuged at 5000 rpm for 10 min at 4^{o}C and the bacterial cells were removed. The supernatant was directly used for antagonistic test. Pellet was diluted with sterile distilled water and used for antimicrobial test.

The antimicrobial activity of the lactic acid bacteria was determined by using diffusion method as described by Sandra et al., 2012. The plates were poured with 20 mL nutrient agar. The overnight cultures of pathogenic strains were spread on the surface of nutrient agar. The agar plates inoculated with pathogens were incubated for 1 h before making wells on the plates. 30 µL of the extract and10 µL of respective pellets were added to the wells for screening of antimicrobial activity.The plates were kept at 4^{o}C for 30 minutes to permit diffusion of the assay material and incubated for 24 h at 37^{o}C. Zones of inhibition were measured(Hoseini, 2004).

The colonies that passed all the three criteria were subjected to catalase test and gram staining. Gram positive and catalase negative isolates were sub cultured in MRS broth and stored at -20^{o}C for further sugar fermentation and salt tolerance test.

3.2.2Preparation of yoghurts and their evaluation

Selected potential probiotic isolates from *nak cheese* (**R-5, J-8 and S-7**) and cow cheese(**P-5 and P-6)**were used for the preparation of two yoghurts. Bifidobacterium animalis; BB-21 used by DDC, Nepal for production of probiotic yoghurt was used to prepare yoghurt in the same environment and condition for comparative analysis of the product (Fig 3.2).

Raw materials/process	Notes
Milk	Cow Milk
↓	
Pasteurization	85°C for 30 minutes
↓	
Homogenization	contents mixed uniformly with a glass rod and Milk placed in plastic cups
↓	
Milk cooled	Temperature brought to 42°C
↓	
Culture inoculation	4 µL/ 100 mL pellet of overnight culture and cups covered with aluminum foils
↓	
Holding	at 42°C for 8 h
↓	
Cooling	cooled to 4°C to stop the fermentation process

Figure 3. 2: Different process involved in laboratory preparation of yoghurt

1. Titrable acidity and pH determination

pH was measured by calibrated pH meter. Acidity was measured by titrating the 1:4 diluted yoghurt against 0.1 M NaOH, using Phenolphthalein as an indicator.

2. Quantification of total solid content

2 g of yoghurt was placed in a glass plate and dried in hot air oven at 121^{O}C until the content on the plate formed a dried mass. Finally the differences between the empty glass plate and glass plate with the solid was used for determining the solid content.

3. Organoleptic evaluation

A panel of 10 untrained judges consisting of 3 females and 7 males were taken for sensory evaluation of the three yoghurt. 9 points Hedonic scale was used for both quantitative and qualitative evaluation of the products and to find the overall acceptance of Yoghurt attributes; Overall acceptability, texture, flavor and taste. The individuals taken were healthy, willing to volunteer and had no any history of intolerance to yoghurt.

Procedure- All testing was performed one-on-one basis. Judges were brought to sit at the table. The yoghurts were presented side by side on a small serving plate, and the instruction for the use of the hedonic scale was given. They tested the samples based on the following

attributes: flavor, texture and taste. Responses were given using the 9- point's hedonic scale (Table 8.1). The scale was structured, it had labels but on numbers. Judges tried the first sample and responded by marking on appropriate position on which the labels for 9-points hedonic scale were printed. Judges then tasted remaining samples and responded in the same way(Ihekoronye and Ngoddy, 1985). Overall acceptance of the products were calculated using the mean scores of the attributes.

3.2.3. Shelf-life of yoghurts

1. Stability of the lab-prepared yoghurts

Yoghurts prepared using cheese isolates and*Bifidobacterium animalis*; BB-21was refrigerated at 4^{o}C for 7 days. The $_{P}$H of these samples were measured on the 1st, 3rd, 5th and 7th day respectively for observing the trend of their $_{P}$H change.

2. Viability of the strains used for yoghurt preparation

Pour plate of 10^{8} dilution of the yoghurts prepared in lab was carried out on 1st, 3rd, 5th and 7th day of refrigeration. Colony count was carried out to determine the maintenance of >6log cfu/g of the bacteria in yoghurt.

3.2.4 Phytochemical analysis of plants used as fodder for nak.

1. Preparation of plant extract

In order to perform phytochemical analysis, dried leaves and stems of the sampled plants *Rhododendron lepidotum, Galtheria nummularioides and Artemisiavulgaris*were grinded and extraction solvent methanol was added as solvent (750mL per 100g of dry sample). Sonication was carried out for three days. Then the extracts were filtered under vacuum and concentrated at reduced pressure using a rotary evaporator at 37^{o}C. The dried extracts were kept in the refrigeratorat 4°C until use (Sethi et al., 2013).

2. Phytochemicalsscreening

All the screening tests for the phytochemicals were carried out using methods described by Katasaniet al., 2011.

a. Flavonoids:

Alkaline reagent test: To the 5 mL test solution, 2mL of 2% NaOH was added. Formation of an intense yellow color, which turns to colorless on addition of few drops of dilute acid indicated presence of flavonoids.

b. Tannins:

Ferric chloride Test: About 1 mL of the ethanol extract were added in 2 mL of water in a test tube. 2 to 3 drops of diluted ferric chloride solution were added and visually observed for green to blue-green (cathechictannins) or a blue-black (Gallic tannins) coloration. .

c. Saponins:

Frothing Test. The extract of 50 mg were first dissolved in 1 ml water and filtered. The filtrate was diluted with 4 mL distilled water. The mixture was shaken vigorously and then visually observed for a stable persistent froth.

d. Phenols:

Wagners test: 20 ml of ethanol extract was evaporated the dry residue will be dissolved in 5 ml of HCl (2N) and filtered. A few drops of Wagner was added, the presence of red coloredprecipitate indicated the phenols.

e. Steroids and terpenoid:

Salkowski test; Five mL of the extracts were treated with 2 mL of chloroform with 3 mL of conc. Sulphuric acid. The mixture was shaken well and allowed standing for some time. Red color appeared at the lower layer indicated the presence of steroids and formation of yellow colored lower layer indicated the presence of terpenoid.

3. Phytochemical analysis of cheeses

Above mentioned procedures of phytochemical analysis were followed for Methanolic extract of *nak*cheese and cheese from cow's milk as well. Spectrophotometric quantification of the phytochemicals was conducted.

4. Antibacterial activity of phytochemicals by agar well diffusion method

Sensitivity of bacterial strains to various phytochemical extracts of cheese and plants were measured in terms of Zone of inhibition using agar well diffusion assay as described byShakouieet al.,(2012). Bacterial strains used in this study such as *Escherichia coli* and *Staphylococcus aureus*. The bacterial cultures were grown in Muller Hinton Agar and Muller Hilton Broth. The sterile plates containing Muller-Hilton agar medium were spread with fresh bacterial culture by using sterile cotton buds. Well (5 mm size) were made from agar plates by using sterile corkborer, the wells were loaded with 30 µl of phytochemical extracts of concentration (25, 50 and 100 mg/mL). The plateswere incubated at 37^{o}C for 24h. After, inhibition the plates were observed for the presence of clear inhibition zone around the well. The zonesof inhibition were calculated by measuring the diameter of the inhibition zone around the well. Methanol and Ampicillin were used as controls.

5. Determination of total phenol content

Total Phenolic Content was determined by using Folin-Ciocalteu method with Gallic acid as standard as described by Gao, 2000. In brief, 100µL of each extract solution (2.5mg/mL) was mixed with 1mL of Folin reagent. After standing for 3 minutes, 0.8 ml of 1M Sodium Carbonate was mixed and shaken. The mixture was allowed to stand for 1 hour and the absorbance was measured at 765 nm. The calibration curve was prepared using Gallic acid as the standard of concentrations 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L, 150 mg/L , 175 mg/L , 200 mg/L ,225 mg/L and 250 mg/L. Total phenol values were expressed as mg equivalent per gram dry extract weight.

6. Determination of total flavonoid content

Aluminum chloride colorimetric method as described by Gao, 2000,was used for flavonoids determination with some modification. In brief 0.25mL of each extract solution (10mg/mL) was mixed with 1.4 mL of distilled water. 0.75 mL of methanol was added then 0.005 mL of 1M Potassium Acetate was added. Then after 5 minutes, 0.005 mL of 10% Aluminum Chloride was added .The mixture was shaken and the absorbance was measured at 415 nm using UV spectrophotometer. The calibration curve was prepared using quercetin as the standard of concentration 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L, 50 mg/L, 60 mg/L, 70 mg/L, 80 mg/L, 90 mg/L and 100 mg/L. Total flavonoid values were expressed as mg quercetin equivalent per gram dry extract weight.

7. Determination of antioxidant activity using 2, 2- diphenyl-1-picrylhydrazyl free radical

Antioxidant activity of the cheese extracts was assayed using DPPH free radical (Kim et al., 2007) with modification. DPPH solution (0.2 mM) was prepared by dissolving 7.886 mg of DPPH in 100 mL methanol and Stirred overnight at 4^{o}C. Thus prepared purple colored DPPH free radical solution was stored at -20^{o}C for further use. Different concentrations (100, 200, 300, 400, 500, 600, 700 and 800 µg/mL) of methanolic solutions of each extracts were prepared by the serial dilution of the stock solution (10 mg/mL) of the respective extract. To each 0.5 mL extract solution, 0.5 mL of 0.2 mM DPPH solution was added. A control was prepared by mixing 0.5 mL methanol and 0.2 mM DPPH solution. These samples were shaken well and kept in dark for 30 minutes at room temperature. The absorbance was measured at 517 nm against the blank solution consisting MeOH. The radical scavenging activity was expressed as the radical scavenging percentage using the equation

% Radical Scavenging Activity= [(Control Abs- Sample Abs)/ Control Abs] x100

IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the graph of radical scavenging activity against the concentration of

extracts. Statistically, the correlation between antioxidant activity and total phenolic content was determined by plotting IC_{50} (µg/mL) against TPC (mg/g).

8. Influence of cheese components on probiotic bacteria

In order to determine the effect of components in cheese to the viability of lactic acid bacteria (i.e determine the positive or negative effects of antioxidants in cheese extract on lactic acid bacteria)OD at 550 nm of the nutrient media with bacteria only and nutrient broth supplemented with extract of the cow and two Nakcheese with high antioxidant activity was compared with modification as described by Chodak et al., 2008.

Control consisting of 6 mL nutrient broth and 4 µL pellets of the overnight culture of each of the fourprobiotic isolates; dissolved in sterile distilled water, was incubated at 37^{o}C and in a culture tubes and OD was measured at 550 nm at 0, 2, 4 and 24 h respectively. Similar step was followed for Nutrient broth with cow cheese and Nak cheese extract (100mg/mL) together with theirProbiotic isolates. The change in OD indicating the change in bacterial population upon incubation at different time intervals was compared.

3.2.5 Brine shrimp lethality bioassay of cheese extract.

Hatching the brine shrimp

Brine shrimp eggs (*Artemia salina*) were hatched in artificial salt water prepared from salt (40 g/L) and supplemented with 6 mg/1 dried yeast. The two unequal compartments plastic chamber with several holes on the divider was used for hatching. The eggs were sprinkled into the larger compartment which was darken, while the smaller compartment was illuminated. After 48 h incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighted side whereas their shells were left in another side (Solis et al., 1993).

A. Bioassay

The procedure for BSLT was modified from the assay described by Solis et al., 1993. Stock of extract of concentration 2.5 mg/mL was made and serial dilution was carried upto 10^{-1} and 10^{-2}. 100µL of respectively each of stock and the serially diluted test extract were placed in the wells of 96-well microplates. 100µL of suspension of nauplii containing 10-15 organisms was added to each well so that the final concentration was 1000 µg/mL, 100µg/mL and 10 µg/ml. Potassium dichromate was used as positive control and salt water with shrimp only was taken as negative control. The plates were covered and incubated at room temperature (25-29°C) for 24 h. Plates were then examined under the binocular stereomicroscope and the numbers of dead (non-motile) nauplii in each well were counted. 100 µL of methanol were then added to each well to immobilize the nauplii and after 15 minutes the total numbers of brine shrimp in each well were counted. The percentage

mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.The lethal concentrations of cheese extract resulting in 50% mortality of the brine shrimp (LC_{50}) from the 24 h countswas calculated from the graph of % mortality against the log of concentration of extracts .

3.2.5 Statistical analysis

All experiments were carried out in triplicates.One way and two way ANOVA was carried out for data analysis at 0.05 level of significance using Microsoft Office Excel 2010. Calculation of linear correlation coefficient and correlation analysis were carried out using MS office Excel 2010.

CHAPTER IV

RESULTS

4.1Total LAB count and probiotic enumeration

Colony count by quadrant method was performed for the enumeration of total lactic acid bacteria and total probiotic bacteria in the cheese samples. The colonies with circular clear zone as described by Valerie Coeuret, (2006) were counted as lactic acid bacteria.Cow cheese (1900 m) with ripening time of a month gave the highest total bacterial count of$3.31\times10^6\pm16.1$cfu/gand*nak* cheese (2400 m) gave the lowest total count of 9.6×10^5 ±9.50 cfu/g(Table 4.1).White colonies with circular clear zone in MRS media with 0.25% bile salts were counted as probiotic bacteria. Table 4.1 shows the average of the total probiotic bacteria with standard deviation as experiments were carried out in triplicates.

Table 4. 1: Total LAB count (cfu/g) on MRS agarand total Probiotic enumeration (cfu/g) on MRS agarwith 0.25% bile salts of cheese samples obtained by colony count method.

Cheese	**Total LAB count(cfu/g) (30^oC, 48 h)**	**Total Probiotic count(cfu/g) (37^oC, 72 h)**
Cow cheese (1900 m)	$3.3\times10^6\pm16.1^a$	$1.0\times10^5\pm1.0^d$
Nak cheese (2400 m)	$9.6\times10^5\pm14.5^d$	$2.2\times10^5\pm2.4^c$
Nak cheese (2600 m)	$1.1\times10^6\pm13.0^b$	$3.2\times10^5\pm1.2^b$
Nak cheese (2900m)	$1.8\times10^6\pm9.5^c$	$3.6\times10^5\pm2.2^a$

Each Value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$).

The *nak* cheese (2900 m) gave the highest Probiotic count of 3.6×10^5 ±2.2 cfu/g and Cow cheese (1900 m) gave the lowest count of 1.0×10^5 ±1.0cfu/g.*Nak* cheese compared to cow cheese had greater total probiotic count. Cow cheese having short ripening time and highest of the total bacterial count comparatively, had lowest of the total probiotic population (Table 4.1).

The total probiotic count of all the four cheeses were lower than 10^6 cfu/g There was a significant difference in total lactic acid bacteria count and total probiotic count of the cheese ($p<0.05$). Total probiotic bacteria in the cheese were significantly lower compared to the total lactic acid bacteria present in it. Of the total lactic acid bacteria present or added to the cheese only minimal number of bacteria possessed probiotic properties.

4.1.1 Effect of ripening duration on microbiology of cheese

Nak cheese (2400 m) ripened for 1, 3 and 4 month respectively was studied for the effect of ripening period on cheese microbiology. Considering the bacterial population of cheese starter culture to be >8log cfu/g which is a suggested minimal and ignoring the natural presence of lactic acid bacteria in cheese milk or their accidental presence, a significant negative linear relationship existed between the ripening time of cheese and total bacterial count (Fig 4.1) at r (1) =0.99, $p<0.05$ ($\alpha=0.05$). The bacterial population in the cheese decreased with increased ripening period.

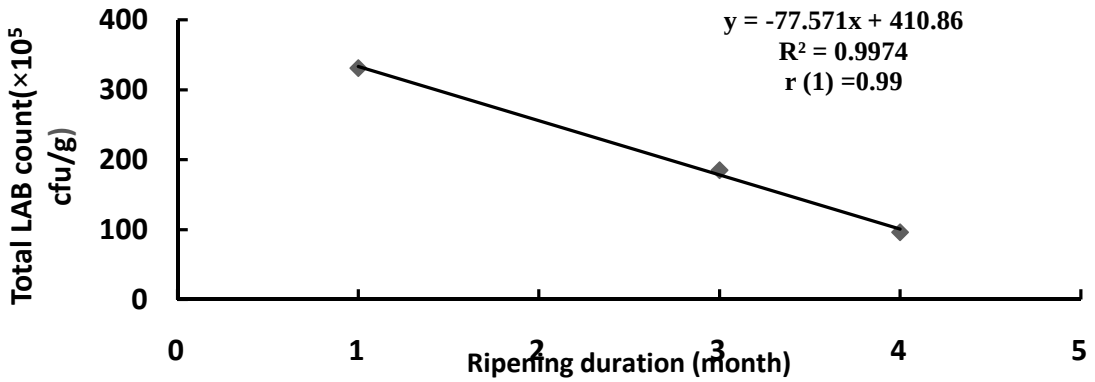

Figure 4. 1: Linear correlation between ripening time of the cheese and total LAB count.

4.2Selection of gastro-intestinal (GI) probiotics

Total 32 colonies from the four cheese samples obtained in MRS media with 0.25% bile salts were picked, sub cultured and assayed for their probiotic properties. The isolates were subjected to acid tolerance test, bile tolerance test and their supernatant and cell pellets were tested for antimicrobial activities against three indicator organisms; both gram positive and gram negative respectively (Table 8.3)**.** Of the 32 isolates 1 survived pH 2 and 21 survived PH 3 for 3 h. Out of 21 isolates only 17 were able to survive bile concentration of 0.25% for 4 h but none survived the concentration of 0.3% for 4 h. Of the total 17 isolates taken for antimicrobial assay only 12 showed inhibition to all the three indicator organisms. Only12 of them i.e. 37.5 % were able to survive pH 3 for three hours, 0.25% bile salts for four hours and showed inhibitory activities upon both the gram positive and gram negative indicator organisms with diameter of inhibition zone greater than 7 mm (Fig 4.3). 3.1 % of the isolates were able to survive pH 2 for three h.Remaining 20 isolates were discarded due to their failure to meet all of the three criterion for probiotic selection. Most of the probiotic bacteria showed strong antimicrobial activities upon gram positive organisms compared to gram negative microorganisms.

Of the total 32 isolates taking 8 from each of the four cheese samples , *nak* cheese (2400 m) gave the highest number 12.5% of probiotics and Cow cheese (1900 m) the lowest i.e. 6 % (

Fig4.2)that meet the criterion to be used in dairy products for human beings. There was a significant difference in the total probiotic population of the cheese during enumeration and the probiotic population meeting all the three selection criteria along with the time factor (P< 0.05). The latter probiotic population was lower than the former.

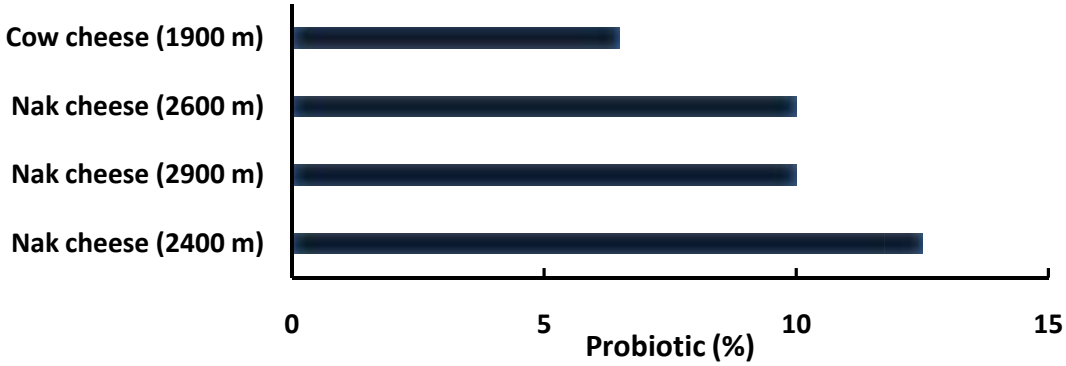

Figure 4. 2: Percentage of probiotic LAB in different cheese samples meeting the criterion to intestinal probiotics.

Figure 4. 3: Antibacterial Test of LAB against *E. coli* (left),*S. aureus* (middle) and *P. aeruginosa (*Left*)* on nutrient agar cultured overnight at 37^{o}C.

4.3Presumptive identification of isolates

4.3.1 Morphological study

The morphological observation of 8 isolates showed spherical, creamy white and smooth colonies. Gram staining followed by microscopic view revealed all the isolates to be gram positive and cocci in chain and pair resembling to *Streptococcus and Lactococcus*. (Table 4.2).

Table 4. 2: Colony characteristics and Gram staining of isolates.

S.No	Isolates	Colony Characteristics	Gram staining	Probable Phenotype
1	**R-5**	Spherical, creamy white, smooth	Positive, cocci in chain and pairs	*Streptococcus, Lactococcus*
2	**R-7**	Cylindrical, creamy white, smooth	Positive, cocci in chain and pairs	"
3	**J-1**	Cylindrical, creamy white, smooth	Positive, cocci in chain and pair	"
4	**J-7**	Spherical, creamy white, smooth	Positive, cocci in chain and pair	"
5	**P-5**	Cylindrical, creamy white, smooth	Positive, cocci in chain and pair	"
6	**P-6**	Spherical, creamy white, smooth	Positive, cocci in chain and pair	"
7	**S-3**	Cylindrical, creamy white, smooth	Positive, cocci in chain and pair	"
8	**S-7**	Spherical, creamy white, smooth	Positive, cocci in chain and pair	"

Here isolates code; S-, R-, J- and P- stands isolates of *nak* cheese (2900 m), *nak* cheese (2400 m), nak cheese (2600 m) and cow cheese (1900 m) respectively.

4.3.2 Biochemical Test

All the 8 isolates subjected to catalase test, were found to be catalase negative confirmed by no observation of gas bubbles on isolates smeared slides with Hydrogen Peroxide (Table 4.3).The isolates were thus anaerobic in nature. Similarly the 8 isolates were salt intolerant (4% NaCl) and gave positive lactose and galactose fermentation test carried out in nutrient broth with 1 % of the sugars and Phenol red as indicator during 24 h of incubation at 42^{o}C. In positive test for sugar fermentation the color of the broth changed to orange from red due to production of organic acids. Based on the biochemical test these isolates were presumptively identified as *Streptococcus thermophilus.*

Table 4. 3: Catalase test, Sugar fermentation and NaCl tolerance of the isolates at 42^{o}C.

Isolates	Catalase test	Sugar fermentation test		NaCl (4%) tolerance
		Lactose	Galactose	
R-5	-ve	+ve	+ve	-ve
R-7	"	"	"	"
J-1	"	"	"	"
J-7	"	"	"	"
S-3	"	"	"	"
S-7	"	"	"	"
P-5	"	"	"	"
P-7	"	"	"	"

Here isolates code; S-, R-, J- and P- stands isolates of *nak* cheese (2900 m), *nak* cheese (2400 m), nak cheese (2600 m) and cow cheese (1900 m) respectively.

4.4Product development and comparative evaluation

Three yoghurts viz; Yoghurt I (*Nak* cheese isolates: **R-5, J-8 and S-7**), Yoghurt II (Cow cheese isolates; **P-5 and P-6)** and Yoghurt III (*Bifidobacterium animalis; BB-21*) were prepared under similar laboratory conditions. The yoghurts were creamy white in color, had smooth texture and pleasant smell (Table 4.4). All the three yoghurts had acidity within the standard of >0.7 % (as lactic acid) as suggested by FDA (2009). The pH of BB-12 yoghurt was less than 4, which is as expected and the pH of the yoghurts prepared from cheese isolates (*Streptococcus thermophilus*) were within the limit of 4-5 as suggested. The total solid content of the yoghurts within the range 11.07-11.90 %, which was lower than 13.6- 18.8 % for plain yoghurt as reported by Dublin-Green and Ibe (2005).

Table 4. 4: Physical characteristics of yoghurt sample prepared from different combination of probiotic cheese isolates and commercially used probiotic LAB (*Bifidobacterium animalis: BB-21).*

	Yoghurts		
Characteristics	**I**	**II**	**III**
Color	Creamy white	Creamy white	Creamy white
Texture	Smooth	Smooth	Smooth
Smell	Pleasant	Pleasant	Pleasant
Taste	Sweet	Little sour	Little sour
Ph	4.46 ± 0.07^{a}	4.38 ± 0.06^{b}	3.72 ± 0.03^{c}
Acidity (% as lactic acid, m/v)	1.17 ± 0.06^{c}	1.20 ± 0.02^{b}	1.29 ± 0.04^{a}
Total Solid (%)	11.07 ± 0.04^{c}	11.19 ± 0.07^{b}	11.90 ± 0.1^{a}

Each value is the average of three values ± standard deviation. Values with different superscript within the same row are significantly different ($p<0.05$). Here Yoghurt I, II and III are prepared from*nak*cheese isolates (**R-5, J-8 and S-7**), cow cheese isolates (**P-5 and P-6**) and commercial BB-21 respectively.

4.4.1 Organoleptic evaluation

Three different yoghurts were prepared under similar laboratory conditions and subjected to organoleptic evaluation, where each physical attributes were scored by the judges from 1-9. Mean scores were calculated and each score were related to the 9-point hedonic scale. The three types of yoghurt were not significantly different in overall acceptance, flavor, texture and taste ($P>0.05$). But when the attributes were taken as variable, there was a significant difference between them, within a sample at $p<0.05$. From figure 4.4, Yoghurt I was rated highest for taste, overall acceptance and texture but was not significantly different from other yoghurts ($P>0.05$). Yoghurt III was rated highest for flavor but was not significantly different from other yoghurt ($P>0.05$). Among the yoghurts prepared from cheese isolates, Yoghurt I (*nak* cheese isolates) was scored higher for overall acceptance than yoghurt II (cow cheese isolates).

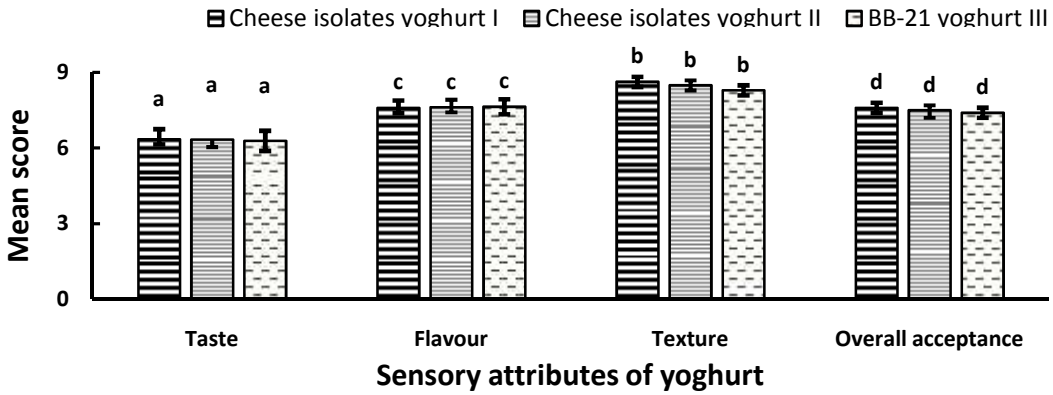

Figure 4. 4: Effect of probiotic cultures on sensory attributes of yoghurt.

Values are means ± SD triplicate determinations, Values with different superscript are significantly different ($p<0.05$).Here Yoghurt I, II and III are prepared from 3 *nak*cheese isolates, 2 cow cheese isolates and commercial BB-21 respectively.

4.4.2 Shelf life of yoghurts

a. Stability of probiotic yoghurt

Within three yoghurt samples the change in pH for each day was significantly different, $p<0.05(\alpha=0.05)$. Total decrease in pH of each of the yoghurt for seven days was significantly different.Yoghurt III had a higher decrease by 18 % but within acceptable limit compared to yoghurt prepared from cheese isolates**.** Overall the three yoghurts remained stable for seven days of refrigeration with a decrease in pH by 11-18 % (Fig4.5). Comparatively the stability of yoghurts prepared from S. thermophilus were higher than yoghurts prepared from BB-21 strains during 7 days of refrigeration.

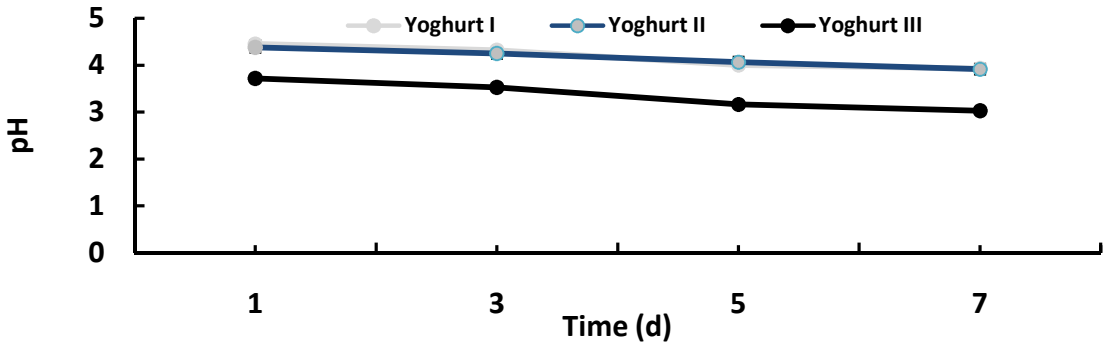

Figure 4. 5: Stability of yoghurt under refrigeration at 4^{o}C for seven days in terms of change in pH.

b. Viability of the bacterial culture in the yoghurt

Total colony count of the yoghurt samples were carried out for seven days of refrigeration. Within three yoghurt samples the decrease in viability for each day was significantly different, $p<0.05(\alpha=0.05)$. Total decrease in viability of each of the yoghurt for seven days was significantly different. There was a huge plunge in the microbial population during seven days of refrigeration but within the prescribed criteria for microbial population of >6log for any dairy products. The cheese isolates remained viable and maintained >6logcfu/g in the product even though the total viable population was lower than the commercially used BB-21 strain (Fig 4.6).

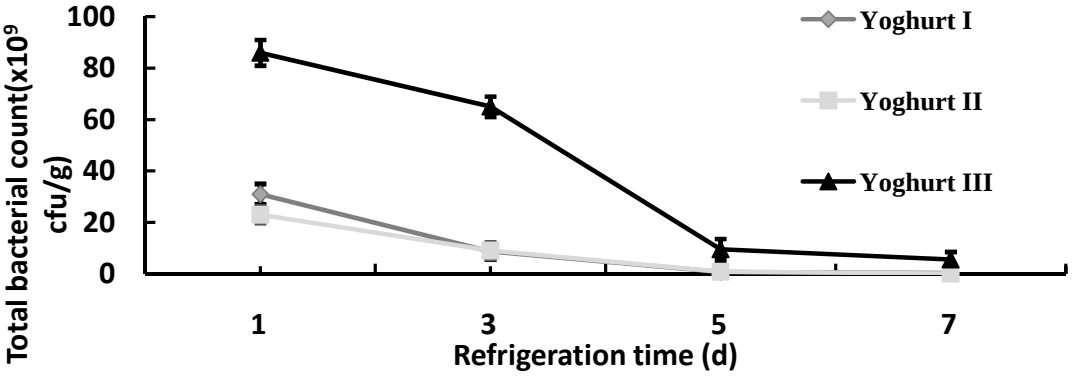

Figure 4. 6: Total bacterial count of yoghurt prepared from cheese isolates (I and II) and BB-21 (III) under refrigeration at 4^{o}C for seven days.

4.5Phytochemical analysis of medicinal plant and cheese.

Steam and leaf of the three medicinal plant specimens foraged by *nak* collected from Rasuwa (2400 m)were used for phytochemical extraction using methanol as a solvent for extraction**.** Similarly methanol was used for preparation of cheese extract as well, following the procedures similar to plants**.**

4.5.1 Yield of extract

The yield of extract in methanol ranged from 1.78 -2.37% for plant specimen considering 5% moisture content and 6.67-7.77 % for cheese considering 20% moisture content (Table4.5). Yield percent was expressed per 100 g of dry weight. The extract yield between plant and cheese was significantly different at $p<0.05$. Cheese gave higher yield percentage compared

to the plant given as fodder. *Nak* cheese (2600 m) gave the highest yield of extract compared to other cheese and plant as well.

Table 4. 5: Extract yield (%) of different plant specimen and cheese sample in methanol.

Sample	Weight of sample taken(g)	Yield (%)
Plants		
Rhododendron lepidotum	4	2.37 ± 0.1^{e}
Galtheria nummularioides	3	2.10 ± 0.2^{f}
Artemisiavulgaris	11	1.78 ± 0.1^{g}
Cheese		
Nak cheese (2400 m)	35	6.67 ± 0.1^{d}
Nak cheese (2900 m)	35	7.13 ± 0.2^{c}
Nak cheese (2600 m)	35	7.77 ± 0.1^{a}
Cow cheese (1900 m)	35	7.62 ± 0.2^{b}

Each Value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$).

4.5.2 Phytochemical screening

The plant and cheese extract were subjected to various phytochemical screening test (Table 4.6). Plant extract gave positive test for phenolic, tannins, saponins, sterols and flavonoids. Cheese extract gave positive test results for phenolic; Wagners test and flavonoids; alkaline reagent test but didn't show the presence of sterols and showed negligible presence of tannins and saponinscompared to plant. The positive test for phenolic andflavonoids enabled to proceed to quantitative estimation of phytochemicals present in cheese extract**.**

Table 4. 6: Phytochemical screening of plant specimen and cheese sample.

	Phytochemicals				
Sample	**Phenol**	**Tannin**	**Saponin**	**Sterols and Terpenoid**	**Flavonoid**
Plants					
Rhododendron lepidotum	++	++	++	++	++
Galtheria nummularioides	++	++	++	++	++
Artemisia vulgaris	++	++	++	++	++
Cheese					
Nak cheese (2400 m)	+	+	+	_	+
Nak cheese (2900 m)	+	+	+	_	+
Nak cheese (2600 m)	+	+	+	_	+
Cow cheese (1900 m)	+	+	+	_	+

(Here, ++ indicates presence of components in significant amount, + indicates presence in trace amount, – indicates absence of component).

4.5.3 Antibacterial activity of extracts

Antibacterial activities of plant and cheese extracts of different concentration (25, 50 and100mg/mL were assayed using indicator organisms *E.coli* and *S. aureus* and observed for inhibition zone. Plant extract showed antibacterial activities for both the organisms at all three concentration (Table 4.7). Antibacterial activities was not observed for cheese extracts at all three concentration for both the indicator organisms (Fig 4.7)**.**

Table 4. 7: Antibacterial activity of plant and cheese extract against *E.coli* and *S. aureus*.

Extract Concentration(100mg/ml)	**Test organisms**	
	E. coli	***S. aureus***
Plant Extract		
Rhododendron lepidotum	**+**	**+**
Galtheria nummularioides	**+**	**+**
Artemisia vulgaris	**+**	**+**
Cheese Extract		
Nak cheese (2400 m)	**-**	**-**
Nak cheese (2900 m)	**-**	**-**
Nak cheese (2600 m)	**-**	**-**
Cow cheese (1900 m)	**-**	**-**

Figure 4. 7: Antibacterial test of different concentration of plant (right) and cheese extract (left) against *E. coli* and *S. aureus* grown in Muller Hilton Agar taking ampicillin as positive control.

4.5.4 Total phenol and flavonoid estimation

Using the equation y=0.005x+0.3907(Fig 8.3) and formula for mg Gallic acid equivalent/ g. of extract= c.v/w. Where c is the concentration of gallic acid from the equation, v is volume of extract used during assay and w is dry weight of extract used in the assay, the total phenolic contents in the examined plant and cheese extracts was

determinedspectrophotometricallyat 765 nm. The values obtained for the total concentration of phenols were expressed as mg of GA/ g of dry sample (DS) for plant and mg of GAE/ g of dry extract (DE) for cheese. (Table 4.8).The total phenol contents in the examined plant extract ranged from 67-112mg of GA/g of dry sample and for examined cheese extract ranged from 2.75-10.9 mg of GA/g of dry extract. Plant extract had significantly higher phenol contents compared to cheese ($p<0.05$).The highest of the phenolic content was shown by *nak* cheese (2900 m) 10.90±0.7 mg of GA/g dry extract and lowest by cow cheese (1900 m) 2.75±0.4 mg of GA/g dry extract(Table 4.8). The total phenol content of *nak* cheese was comparatively higher than cow cheese. Among the three *nak* cheeses studied phenol content varied, ranging from 6.06±0.6 mg GA/g dry extractof*nak* cheese (2400 m)to (10.9±0.3 mg GA/g dry extract of *nak* cheese (2900 m).

Using the equation y=0.0098x-0.1037(Fig 8.4) and formula for mg QE equivalent/ g dry weight= c.v/w, (Where c is the concentration of quercetin from the equation, v is volume of extract used during assay and w is dry weight of extract used in the assay) the total flavonoid contents in the examined plant and cheese extracts was determined spectrophotometrically at 415 nm. The values obtained for the total concentration of flavonoids were expressed as mg of QE/ g dry sample for plant and mg of QE/ g of dry extract for cheese (Table 4.8).The total flavonoid contents in the examined plant extract ranged from 23-79 mg of QE/g of dry sample and for examined cheese extract ranged from 1.8-7.70 mg of QE/g of dry sample. Plant extract had significantly higher flavonoid contents compared to cheeses ($p<0.05$). The highest of the flavonoid content was shown by *nak* cheese (2900 m) 7.70±0.5 mg of QE/g of dry extract and lowest by cow cheese (1900 m) 1.80±0.3mg of QE/g of dry extract. The total flavonoid content of *nak*cheese was comparatively higher than cow cheese as shown in Table 4.8.

Table 4. 8: Total phenolic content (TPC) and flavonoid content of plant specimen and cheese sample.

Sample	TPC (mg GA equiv./g)	TFC (mg QE equiv./g)
Plants (DS)		
Galtheria nummularioides	67.30 ± 0.2^{c}	23.88 ± 0.4^{c}
Rhododendron lepidotum	112.02 ± 0.3^{a}	79.27 ± 0.3^{a}
Artemisia vulgaris	91.72 ± 0.5^{b}	39.66 ± 0.5^{b}
Cheese (DE)		
Nak cheese (2600 m)	9.79 ± 0.8^{e}	$5.45^{e}\pm0.6^{e}$
Nak cheese (2900 m)	10.9 ± 0.7^{d}	7.70 ± 0.5^{d}
Nak cheese (2400 m)	6.06 ± 0.6^{f}	4.12 ± 0.4^{f}
Cow cheese (1900 m)	2.75 ± 0.4^{g}	1.80 ± 0.3^{g}

Each value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$)

4.5.5 Antioxidant activity

The anti-oxidant activity of different cheese extracts was determined using methanol solution of DPPH reagent. The anti-oxidant activity of the extracts was expressed in terms of 50 % inhibition (IC_{50})**.**

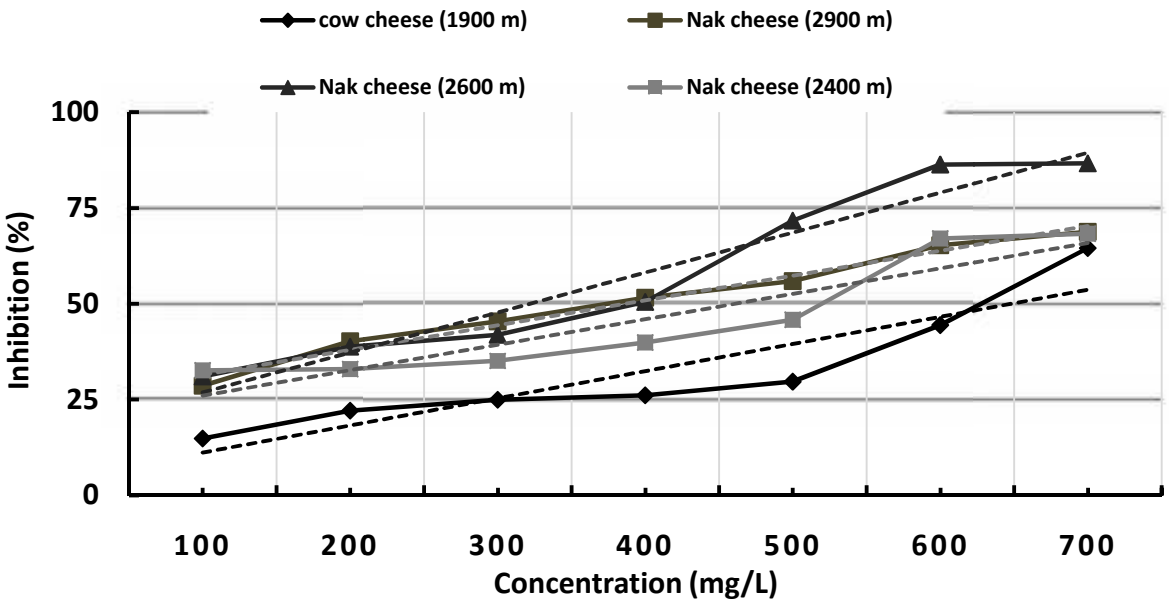

Figure 4. 8: DPPH radical scavenging activity of cheese extract at wavelength of 517 nm.

IC_{50} of cheese

The IC_{50} of cheese extract was determined from the graph of percent inhibition against concentration of the cheese extract (Fig 4.11).*Nak*cheese (2900 m) showed the lowest IC_{50} 371.64±3.3 mg/L implying the strongest antioxidant activities (Table 4.9). Cow cheese (1900 m) exhibited highest IC_{50} 626.24±4.2 mg/L implying the lowest antioxidant activity comparatively.

Table 4. 9: IC_{50} of different cheese.

Sample	IC_{50} (mg/L)
Nak cheese (2900 m)	$371.64±3.3^{d}$
Nak cheese (2600 m)	$394.12±3.1^{c}$
Nak cheese (2400 m)	$518.30±2.7^{b}$
Cow cheese (1900 m)	$626.24±4.2^{a}$

Each value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$).

4.5.6 Antioxidant activity and total phenolic content

Antioxidant activity and total phenol contents of cheeses extracts were in significant positive correlation at r (2) =0.99, p<0.05 (Fig 4.9). The antioxidant activity of the cheese sample increased with the corresponding total phenol content. The cheese with high phenolic content showed high antioxidant activity. The *nak* cheese (2900 m) with higher phenolic content showed higher antioxidant activity compared to other cheese indicating the possible role of phenolic in the increased antioxidant activity.

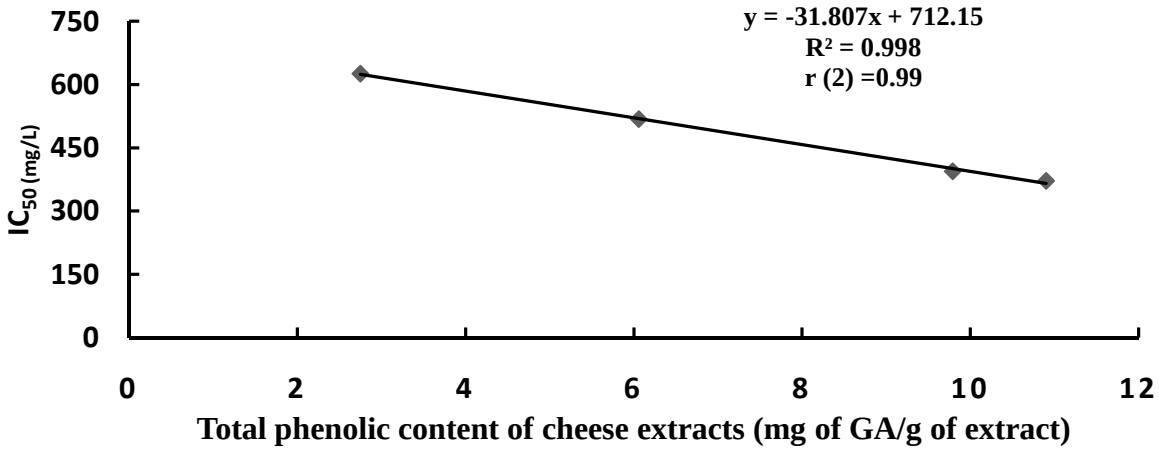

Figure 4. 9: Linear Correlation between DPPH radical scavenging activity and total phenolic content of cheese extracts.

4.6.7Influence of cheese component on probiotic bacteria

The influence of cheese extracton the viability of probiotic bacteria *is* presented inTable 8.2. There was a significant difference in OD at 550 nm between nutrient broth with isolates and cheese extract supplemented nutrient broth with isolates in conjugation with the different time interval of incubation (P<0.05) . The increase in OD of the broth with cheese extract compared to control was observed. The increase in OD observed for 4 h culture ranged from 110- 144% of control to 178-256% for nutrient broth supplemented with antioxidants respectively (Fig 4.10).There were no significant increase in OD obtained from longer incubation times when compared to control. For media with cow cheese extract the percentage increase in OD was lowest compared to media with *nak* cheese extract. The percentage increase in OD was twice than the control after 4 h of incubation. Among the media with *nak* cheese extract, *nak*cheese extract (2900) with higher antioxidant activity gavegreaterincrease in OD compared to *nak* cheese extract (2400 m) and *nak* cheese extract(2600 m) at 4 h of incubation.

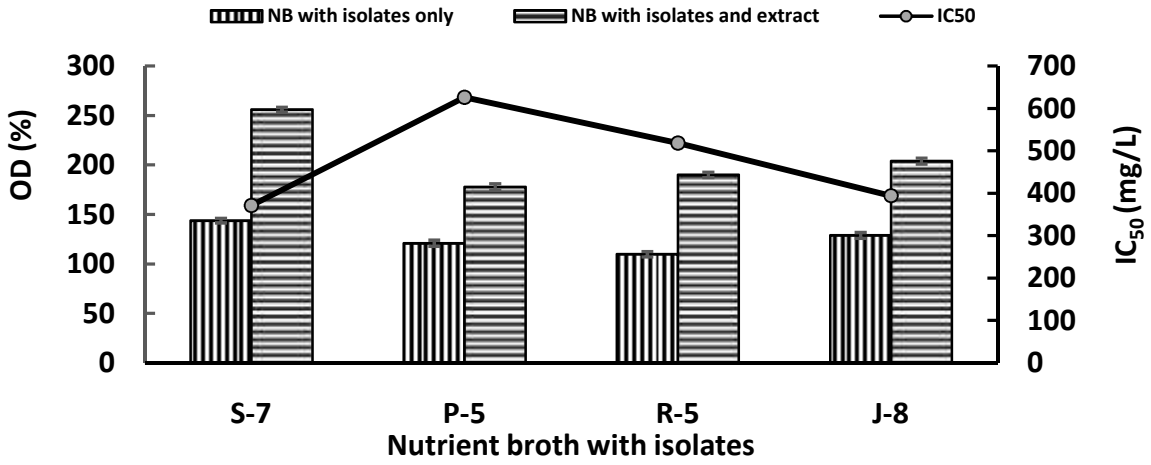

Figure 4. 10. : Percentage increase in OD at 550nm of the nutrient broth with isolates (**S-7, I-5, R-5 and J-8**) only and nutrient broth with isolates supplemented by extract of respective cheese (from which the bacteria were isolated) during 4 h of incubation at 37 ^{o}C.

(Here isolates code; S-, R-, J- and P- stands isolates of *nak* cheese (2900 m), *nak* cheese (2400 m), nak cheese (2600 m) and cow cheese (1900 m) respectively).

4.5.8 Brine shrimp lethality bioassay of cheese extract

In the present study the brine shrimp lethality of extracts of 3 *nak* cheese and 1 cow cheesewas determined using the procedure of (Solis et al.,1993). The LC_{50} of the brine shrimp obtained for extracts of these cheese are given in Table 4.10. The LC_{50} of cheese extract was determined using the formula from the graph of percentage mortality against log of concentration of extract.Methanolic extractof nakcheese (2600 m)showedmost prominent activity with LC_{50} of 350.19±4.6 µg/mL and least activity was of *nak* cheese extract(2400 m) with LC_{50} of 698.95±4.3 µg/mL. The *nak*cheese (2900 m) and cow cheese extract (1900 m) exhibited significant brine shrimp lethality with LC_{50} of 411.183 and 376.84 µg/mL respectively at 24 hours of incubation. Thepercentage mortality was found to be directly proportional to the log of concentration of the extract (Fig 4.12). Maximum mortalities took place at a concentration of 1000 µg/mL ($p<0.05$), whereas leastmortalities were at 10 µg/mL concentration. No significant decrease in mortality existed between concentration of 10 and 100 µg/mL at 24 hours of incubation.

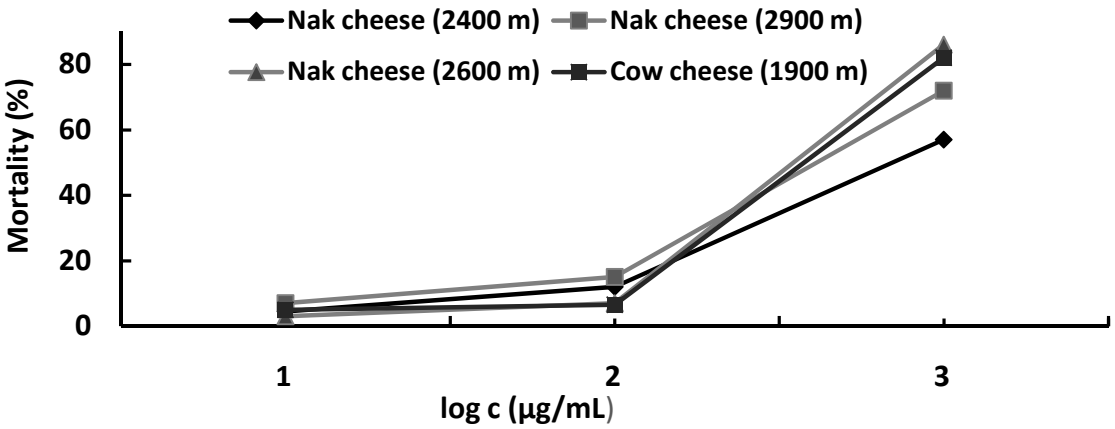

Figure 4. 11: Brine shrimp lethality bioassay of cow and nak cheeses extracts at 24 h.

Table 4. 10: LC_{50} of methanolic extract of cow and *nak* cheese at 24 h of incubation of brine shrimp.

Cheese	**LC_{50} (µg/mL)**
Nak cheese (2400 m)	698.95±4.3[a]
Nak cheese (2900 m)	411.18±6.1[b]
Nak cheese (2600 m)	350.19±4.6[d]
Cow cheese (1900 m)	376.84±5.7[c]

Each value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$)

CHAPTER V

DISCUSSION

Nak cheese were sampled from DDC based factories located at different hilly regions of varying altitudes for a comparative study of the functional aspects of cheese, as the central cheese collection of DDC doesnot differentiate and market the product according to the regions they were produced. A cow cheese was also sampled for a comparative study of the functional aspects of these two types of cheese, owing to differences in the feeding habits and conditions of domestication for cow and *nak*. Cows are farmed at lower altitudes and are mostly given grain based diets and straw as fodder for higher milk production whereas*nak*is nomadic in nature, acclimatized to harsh climatic condition, grazes in the pastures of higher altitudes so encounters variety of green plants of medicinal significance. These factors eventually affect the quality of milk and its nutritional as well as microbiological aspects. Cheese production involves various processes such as acidification, coagulation, cooking, salting, molding, pressing, packaging and maturation or ripening. Many native microflora of probiotic nature may be present in cheese and get affected by such processes as well as milk composition. Therefore it is necessary to monitor the viability and potential of these microorganisms present in cheese. The ripening time of cow-cheese is lower than *nak* cheese as cow's milk is of inferior quality with low fat content (3-4%) and that of *nak* is as high as 7-9 %. Cow cheese are marketed as soft cheese and *nak* cheese are hard cheese, this may also be the reason for the difference in ripening duration.

5.1 Total LAB count and probiotic enumeration

The total lactic acid producing bacteria present in the complex mixture of secondary micro-flora of the cheeses were enumerated by colony count method on MRS (pH 6.2) supplemented with 2-4 % Calcium carbonate.MRS (pH 6.2) is a high and selective media used for isolation of Lactic acid bacteria. The total bacterial count includes yoghurt bacteria, mesophilic bacteria and thermophilic bacteria. The media supplemented with Calcium carbonate allows convenient selection of LAB due to the formation of colonies surrounded by clear zone. The decomposition of Calcium carbonate by organic acid produce Carbon dioxide and water as byproduct and is the cause of clear zone formation along with the creation of anaerobic condition for LAB.

Total probiotic bacteria in the cheeses were enumerated by colony count method on MRS media with 0.25% bile salts.The enumeration of probiotic population in the cheese is important aspect to establish its therapeutic significance conferred by the presence of probiotics. Non-probiotic yoghurt bacteria and mesophilic lactic bacteria commonly interfere with the enumeration of probiotic bacteria. Addition of bile salts into culture media (0.15% w/w) is of the simplest and the most efficient method avoiding growth of non-probiotic yogurt bacteria after incubation at 37°C for at least 72 h as probiotic bacteria

are slow growing (Mortazavian et al.,2006; Vinderola and Reinheimer, 1999). In presence of bile salts, the cell wall of yogurt bacteria is deteriorated, while probiotics are commonly resistant to bile (Mortazavianet al, 2006).The optimum growth temperature for lactic mesophilic bacteria is about 25-31°C (Korbekandi et al., 2011) and reasonably, their ability to growth at higher temperatures is considerably limited by presence of other stress factors such as nutritive substances in media and bile salts (as antimicrobial agents). Incubation of cultures at 37°C would inhibit the growth of mesophilic lactic strains even at bile concentration of 0.15%.The MRS media with bile salts and incubation temperature of 37^{o}C excludes the non-probiotic yoghurt and mesophilic bacteria (Marqueset al., 2011). In this study, the total probiotic count was significantly lower than total LAB count suggesting that 0.25 % bile salts and incubation temperature of 37^{o}C made the media selective for thermophilic probiotics.

The daily dose of viable strain of probiotic bacteria is suggested to be 10^8 – 10^9 cfu (Jayamanne and Adams, 2006). For intended therapeutic significance any product at the end of their shelf life should contain minimum viable probiotic population of 10^6-10^7 cfu/g such that consuming 100-200 g of the product would meet the daily dose (Miller, 2004). But even the total probiotic count of the cheeses did not meet the suggested minimal probiotic population. Therefore *nak* doesn't have functional or therapeutic value of probiotics. The results were similar to the previous studies on swiss-cheese made from raw and pasteurized milks by Dasen and Duboz, (1996), Beuvier et al., (1997) where they reported LAB levels at the end of ripening to be lower than 6log cfu/g in cheese made from pasteurized milk.A significant negative linear relationship existed between the ripening time of cheese and total bacterial count (Fig 3) at r (1) =0.99, $p<0.05$ ($\alpha=0.05$), Considering the bacterial population of cheese starter culture to be >6log cfu/g which is a suggested minimal and ignoring the natural presence of lactic acid bacteria in cheese milk or their accidental presence ripening time tends to have negative impact on the total microbiological load of the cheese. Beresford and Williams (2004) have mentioned that the duration of ripening under controlled temperature and moisture conditions affects the viability of lactic acid bacteria but it is not yet clear to what extent the different LAB strains will survive the long ripening period and to what extent their functional properties will be affected.

5.2 Selection of potential probiotic LAB.

5.2.1 Acid and bile tolerance test.

Total 32 colonies obtained in MRS agar with 0.25% bile salts were further subjected to two in-vitro test that mimic the physico-chemical events occurring in the gastro-intestinal tract. Incubation time chosen for acid tolerance and bile salt tolerance were 3 h and 4 h respectively simulating the residence time in the stomach (Prasad et al., 1998). Most of microorganisms are sensitive to the low pH of gastric juice or alkaline condition of intestine. The LAB also should be able to survive the acid conditions of the stomach and the bile in the upper digestive tract to reach the small intestine (Holzafel et al., 1998). In the stomach bacteria encounters pH as low as 1 and in most in vitro assay pH 3.0 is preferred because at

pH 2.0 there is significant decrease in the viability (Prasad et al., 1998). Although the bile concentration of human intestinal tract may vary but the mean intestinal bile concentration is believed to be 0.3 % (Prasad et al., 1998).

Of the total 32 probiotic bacteria subjected to the in-vitro tests only 17 of them met the criteria suggested. Therefore it cannot be generalized that all probiotic strains are acid and bile tolerant (Clark et al., 1993, Lankaputhra and Shah, 1995). As acid and bile tolerance is strain dependent, care should be taken to select strains based on these attributes, if they are to be used as intestinal probiotics.

5.2.2 Antimicrobial assay

The cell free supernatants and cell masses of the isolates were tested for antimicrobial activity against three pathogenic bacterial strains (*Staphylococcus aureus, E.coli,* and *Pseudomonas aeruginosa)* by using well diffusion method. 12 isolates were able to show antibacterial effect against the indicator pathogens. According to Fuller (1989), the production of antimicrobial compounds such as organicacids, short chain fatty acids and bacteriocins is one of the functional properties used to characterize probiotics.The capacity to produce different antimicrobial compounds is an important characteristics of isolates to competitively exclude pathogen, survive in the intestine and show their probiotic effect in the host (Salminenet al., 1998). The results of the experiment are in agreement with Bassyouni et al.,(2012),Kabuki et al.,*(*2006) and Khalil(2009) for different strains ofprobiotic bacteriatested against *Staphylococcus aureus, E.coli,* and *Pseudomonas aeruginosa* and all of the tested isolates had antibacterial effect against all the pathogenic bacteria.

The result indicates that consumption of dairy products with presence of these isolated bacteria in adequate number can help prevent diarrhea, dysentery and other health disorders caused by the indicator pathogenic bacteria being used in this research. Anti-bacterial activities of LAB due to production of organic acids or anti-bacterial substance is an important aspect of lactic acid fermentation process whereby the shelf-life of food products is extended due to reduction in the incidence of food spoilage organisms. Production of pickles, sauerkraut, cheese etc. are result of Lactic acid fermentation carried out by LAB and these products have longer shelf-life, are healthy and microbiologically safe.

5.2.3 Presumptive identification of isolates

Lactococcus is mesophilic bacteria with optimum growth temperature of 25-31 ^{o}C and most of them do not survive at higher temperature whereas *Streptococcus thermophilus*cantolerate temperature higher than 37^{o}C.Similarly is *Lactococcus*tolerant to 6% salt concentration or higher whereas *Streptococcus thermophilus* are not tolerant to salt concentration greater than 2% or higher. Taking also into account the starter culture used for cheese production (*Streptococcus thermophilus* and *Lactobacillus helveticus*), all the 8 selected LAB with probiotic propertieswere presumptively identified as*Streptococcus*

thermophilus. The lactose and galactose is converted to lactic acid by LAB during fermentation which turns the media with 1 % sugar and phenol-red to orange.Lactose utilization is a common characteristics for *S. thermophilus*whereby lactose is broken to glucose and galactose and lactic acid is produced from glucose by most of *s. thermophilus* which are gal -ve.Galactose positivestrainsare technologically important since they can inhibit the growth of undesirable lactic acid bacteria, prevent browning defects (Giraffa et al., 2001) and also been exploited as a target for metabolic engineering to enhance exo-polysaccharide biosynthesis (Levander et al., 2002). Mukherjee and Hutkins (1994) and Vin et al., (2005) isolated Gal+ *S. thermophilus*from cheese and yogurt samples.

The isolated *Streptococcus* sps showed results of acid tolerance, bile tolerance and antibacterial activities against the pathogens used, similar to *Streptococcus thermophilus* CHCC 3534 strain that was examined for its probiotic efficacy by Khalil (2009). The genes and molecules of *Streptococcus thermophilus* have known to support probiotic action (Lebeer et al., 2008).*S. thermophilus* is one of the most important LAB used in food industry. In spite of that, focus has long been on the incorporation of selected strains of *Lactobacillus* species into milk and fermented products, due to extensive studies performed on their probiotic properties compared to the scare and unconvincing data concerning *S. thermophilus* (Khalil, 2008). In addition, much less is known about the certainity of health promoting effects of several members belonging to *S. thermophilus* and the survival of their cells after passage through the human gastro-intestinal tract (Holzapfel et al., 2001). *S. thermophilus*are esteemed as probiotics, but their ability to attach to the intestinal mucosa is weakbecause they are not the natural inhabitants of gut intestinal flora (Mitchel and Sandine, 1986). Studies have showed that these bacteria have positive health benefits due to their biogenic effect, such as improvement of lactose digestion and elimination of symptoms of lactose intolerance,theyreduce the risks of certain cancers, ulcers, imflammation, stimulate the immune system, and also can be used for curing some atopic dermatitis (Guarner et al., 2005).In the context where there is growing need for new strains of *S .thermophilus* with improved health function than the traditional ones, these species of bacteria meeting the selection criteria of probiotics and showing antibacterial activities could hold industrial significance and represent an interesting candidate for use in biopreservation and in control of food spoilage caused by food borne pathogens.

The *Streptococcus* isolated could be an adventitiously present novel strain other than those added as cheese starter and have passed into the milk from the plants on which the cattle forage and finally have survived the pasteurization temperature to be eventually present in cheese.As stated by Beresford, (2011), even if the cheese starter is defined the secondary flora is composed of adventitious microorganisms gaining access to the cheese either from ingredients or the environment.A recent study conducted at National Dairy Research Institute, India showed that *S thermophilus* isolated from plant sources possess similar physiological and biochemical properties to those from dairy sources and can be considered for developing new starters (Maheswari et al., 2014).The identification of species and strain requires 16srRNA sequencing as biochemical test alone is not sufficient.

Out of 32 isolates subject to morphological observation 24 were cocci and only 8 isolates were rods. The dominant probiotic LAB in the selected cheese secondary microflora were *cocci.*The results are not similar to the data for many of the cheeses studied which mentioned thatthe dominant species during ripening is Lactobacillus (Jordan and Cogan, 1993; Lombardi etal., 1995).Since all the isolates meeting all the probiotic selection criteria were *S. thermophilus* (if starter culture) only this genera retained their probiotic properties or remain viable during long duration of ripening compared to *L. helveticus* (also used as starter). The results also indicate that the isolates were comparatively more resistant to the technological processes involved than other related or non-related species.

5.3 Yoghurts development and comparative study

The cheese isolates met the criteria of probiotics such as stability in acid and bile salts along with the minimal time period of tolerance suggested, production of antimicrobial substances and survival at optimal human body temperature of 37^{o}C as described by Szajewska et al., 2007. Since the isolates are known to weakly colonize the intestine so further investigation is required to confirm their possible probiotic effect on human beings. Studies have reported the biogenic effect of the bacteria. Therefore isolates were used for developing yoghurt of short shelf and comparative study was carried out with commercial probiotic strain (*Bifidobacterium animalis).* .Depending on the standards of hygiene observed during the manufacture of Yoghurt and the microbiological quality of the ingredients the shelf-life of yoghurt is around 3 weeks under refrigerated condition (Tamime and Robinson, 1985).

The yoghurts prepared from Cheese isolatesremained stable over seven days of refrigeration with certain decrease in $_{P}$H which may be attributed to production of various organic acids such as lactic acid and acetic acid. The stability of yoghurts with *Streptococcus thermophilus*were more stable than *Bifidobacterium animalis*The isolates maintained their viability of suggested minimum of 10^{6}-10^{7}cfu/g (Miller, 2004) required for achieving optimal potential therapeutic effects of any probiotics in the product during seven days of refrigeration, even though there was a huge plunge in viability during this storage duration. The organic acid produced by the LAB and other factors such as nutrient deprivation and storage condition affect the viability of bacteria in the product over longer period of refrigeration. But comparatively the viability of *Bifidobacterium animalis was* higher in the yoghurt than Streptococcus *thermophilus.*

Microorganisms present in the cheese have to go through harsh condition of cheese preparation such as coagulation, salting, long period of low temperature ripening. So they are unable to retain their viability in the suggested minimal population. Short shelf life products such as yoghurt can be better option to deliver the health benefits of the probiotics isolated from cheese.

5.3.1 Organoleptic evaluation of lab prepared yoghurts

Yoghurts were prepared from the cheese isolates and a commercial probiotic strain. These yoghurts were subjected to sensory evaluation considering three physical attributes; flavor, texture and taste. The overall acceptance for all the three yoghurt were not significantly different, with Yoghurt I being awarded the highest. Referring to hedonic scale the judges liked very much the texture of all three types of yoghurt, liked moderately the flavorof all three yoghurts and liked slightly the taste of theseyoghurts. However, the rating for the three attributes of the yoghurts differed i.e. when we consider the attributes of yoghurt I, panelist liked slightly its taste but liked very much and liked moderately its texture and flavor respectively.

The cultures isolated from cheese, produced yoghurts withacceptance similar to the yoghurts produced by commercially available probiotic strain. Even though the strains used differ in their genus and if let aside their proclaimed health benefits their products were comparable in terms of their sensory attributes. Therefore, these S. thermophilus used as cheese starter might also be a candidate as a probiotic starter culture.

5.4 Phytochemical analysis of medicinal plant and cheese

Three plants mostly foraged by *nak* and having medicinal importance to the locals were collected on November fromRasuwa. *Rhododendron lepidotum* known as *Sunpati* is used as important ingredient of Tibetan tea and eaten to relieve headache, fever, diarrhea and dysentery, *Galtheria nummularioides* locally known as *Kag ko Ankha*; its leaf is used for treating stomach-ache, swelling and pain and berries for making tea by the locals and *Artemisia vulgaris* or *Pati* is used as antimicrobial during cuts and used dermally as blood coagulant, the juice of the plant is used to treat diarrhoea, dysentery and abdominal pains.

Both stem and leaf were used for phytochemical extraction as *nak* feed the whole plant except root. In this research methanol is used for extraction of phytochemicals from both plant and cheese. Methanol is a good solvent for both polar and non-polar compounds. The efficacy of methanol in extraction of secondary metabolites is higher than other solvent such as water, chloroform and ether and most of the phytochemicals are extracted in methanol (Mohan et al., 2011).

In this research, comparatively higher yield of extract was found for cheese than the plant used for phytochemical extraction. With no related articles on such comparative study possible explanationfor the difference could be the extraction of more polar and non-polar components such as short chain-fatty acids and peptides in cheese than the plants selected for study. The *nak* cheese extract were yellowish compared to extract of cow cheese. Yellow color of cheese extract corresponds to concentration of beta-carotene dissolved in fat, which is associated with exposure to herbage and silage of pasture land as well as temperature dependent denaturation (Martin et al., 2005). The significant yellow coloration of *nak* cheese extract indicates the long-term exposure of *nak*to green vegetation of higher altitudes on average than cow. Cows are mostly fed with grain based

diet for high milk production and are given certain variety of green fodder. Cows spend most of their time tied up in the farms. Whereas *nak* forage freely in the open pastures of higher altitudes and encounter different types of green grass and plants; most of them with high medicinal importance.The results are similar to the findings of Coppa, Verdier-Metz I and Ferlay who have suggested the presence beta- carotene of 100 % grass-fed whole milk to be 4 times higher than the level in milk from conventionally fed cows.

5.4.1 Screening of phytochemicals

The medicinal plant and the four cheeses were screened for the presence of phytochemicals. In this study it was observed that phenols, tannins, flavonoids are present in both plant and cheese extract whereas sterols and saponins were present in plant extract but absent in cheese extract. In previous research work these phytochemicals were present in the selected plant and cheese so the research results are similar. Presence of phytochemicals in cheese might be the result of their transfer from plants to cheese via milk. Hilario et al., (2010) has reported that pasture plants are rich and significant source of bioactive components and they can be transferred into the milk and cheese (Hilario et al., 2010).In recent years. Several studies have shown that some plant compounds (e.g. vitamins,volatile organic compounds) are directly transferred from the grazed herbage to the milk (Fedele et al., 2000: Pizzoferrato et al., 2000). Connel and Fox, 2001 reported that the majority of phenolic compounds found in cow milk are derived from the feed and when the cows are fed large quantities of particular crops. Other phenolic compounds may also be detected in ruminant milk. Moreover some alklphenols present in ruminant milk are derived from phenolics ingested through the animal feed (Kilic and Lindy, 2005). However, the phenols and flavonoids detected in the cheeses might also be produced by secondary microflora of cheese, which may consists of fungus and molds besides LAB. Certain class of flavonoids are produced by fungus as secondary metabolites.Similarly fungi have genes encoding the production of volatile phenols (Chatonnet et al, 1995).

5.4.2 Total phenol and flavonoid estimation

Phenol and flavonoid content of the plants and cheeses were determined by Folin-Ciocalteu method and Aluminum chloride colorimetric method respectively.Phenol and flavonoid can influence milk and cheese taste and can also affect their antioxidant activity. Thus, it was reasonable to determine their total amount in the plants and cheeses extract. Phenols represent one of the major groups of compounds acting as primary antioxidants or free radical terminators. Flavonoids, as one of the most diverse and widespread group of natural compounds, are probably the most important natural phenol. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties.

The presence of phenolic compounds in the milk and later in the cheese might be a result of their transfer from plant to milk (Hilario et al., 2010).

Even though the phytochemicals in the plant foraged by *nak* was high but these chemicals were present in low concentration in the cheese. The phytochemicals when consumed by the animals are metabolized in the cells, are also used in various oxidation process or may be excreted by the body through urine. Similarly the rigorous processing steps of cheese production and pasteurization temperature, as well as interaction between phytochemical and cheese micro flora may also affect the final residual concentration in cheese. The value obtained for TPC of cow's cheese in this study were lower than the value of 577.12 mg GA/100 g obtained by Alyaqoubi et al, (2014) for its raw milk suggesting, the rigorous processing steps of cheese production and pasteurization temperature affects the final residual concentration in cheese. A previous study reported that pasteurization negatively affects the total polyphenol and flavonoid concentration because of the possible denaturation of phenol content and amino acid catabolism (Fedele et al., 2006).The cheese collected for this research were made from pasteurized milk therefore the obtained phenol and flavonoid concentration could be significantly lower than in the milk of respective animals.Certain class of flavonoids are produced by fungus as secondary metabolites. Similarly fungi have genes encoding the production of volatile phenols (Chatonnet et al, 1995). Secondary microflora of cheese is undefined and the fungus or molds present in microflora of studied cheeses might also contribute to their total phenol and flavonoid content. However, the cheese starter culture are not known to produce any such compounds but there are research reports on these LAB metabolizing or degrading these phytochemicals in foodas a metabolic strategy for adapting to environmental hostile niches .

Comparatively, *nak* cheese had higher content of phenol and flavonoid than cow cheese, which may be due the difference in their feeding habit and domestication. The results are also supported by previous study of Hilario et al, 2010, that evaluated the presence of bioactive polyphenol compounds in milk of goats grazing on shrubby rangeland vegetation and full-indoor confinement. In the study, he showed that TPC was affected by animal feeding, and its value was higher in the milk of grazing goat than in the milk of indoor goat.The difference in the content of phenol and flavonoid can be attributed to the possible variation infungal microflora between the cheeses studied.

5.5Antimicrobial activity

In the present study, antibacterial activity of methanolic plant and cheese extracts were investigated against two bacterial species (*Staphylococcus aureus* and *Escherichia coli*) at concentration of 25, 50 and 100 mg/mL. Plant extracts had conspicuous zone of inhibition at all three concentration. The antibacterial activities of medicinal plants are attributed to the presence of flavonoids, tannins and steroidal alkaloids. Cheeses extract showed no conspicuous zone of inhibition at all three concentration in both the pathogens. The result indicates that cheese extract even if they have phytochemicals and antimicrobial proteins,

their presence in low concentration andthe bacteria and pH-dependent transformation of antioxidants antimicrobial activity or denaturation of phytochemicals or antimicrobial proteins during pasteurization may be the probable cause of failure to show antimicrobial activity. According to Bingham 2006, Phytochemicals can be transformed by cheese and intestinal micro flora can or metabolize some polyphenols compounds to catechol and other simple phenols; and the derivatives formed in these reactions are characterized by different antimicrobial activities comparing to the precursors.The result suggest that cheese are prone to spoilage pathogens, so there is a need of controlling contamination during cheese production and ripening stages.

5.6Antioxidant activity of cheese extract

The antioxidant activities of the prepared cheese extracts were investigated by DPPH scavenging assay as *2, 2-Diphenyl-2-picrylhydrazyl Hydrate (DPPH) Radical Scavenging Assay* has been widelyused for the antioxidant activity evaluation of food and biological samples. DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) is a nitrogen centered radical havingmaximum absorbance at 517 nm, which gets converted to 1, 1, diphenyl-2-picryl hydrazine on reactingwith hydrogen donating species, *i.e*., antioxidants present in the sample, especially polyphenols (Iqbal, 2012). The ability of these extracts to scavenge DPPH suggest that they might have antioxidants that react withfree radicals to convert them to more stable products and terminate radical chain reaction.

The linear correlation (r (2) =0.99) between phenol content and IC_{50} value indicates that phenol may also contribute to antioxidant activity of cheese along with antioxidant peptides in cheese present through raw milk or produced during ripening process by the microflora (McIntosh et al., 1995),. Researcher Djeridane et al., 2006; Katsube et al., 2004 have shown positive correlation between phenol content and antioxidant activity. While others (Czapeckaet al., 2005) show poor linear correlation or report total antioxidant activity and phenolic content with no comment.With no clear understanding of flavonoids as antioxidant or the mediators of signaling pathway (Williams et al., 2004), it seems irrelevant to associate flavonoid content of cheese extract to their antioxidant activities.

The bioavailability of antioxidants and their antioxidant activity are also affected by various factors such as transformation by intestinal microflora, pH decrease as in acidification process during cheese production, causing transformation of the antioxidants or the high temperature during extraction and pasteurization degrading the peptides into the forms of lower antioxidant potential in a way as mentioned by Chodak et al., *(*2008).

These assay indicate higher antioxidant activity of *nak*cheese possibly due to the presence of phytochemicals or antioxidant proteins of the cheese and those produced by secondary microflora of cheesemight be helpful in preventing the progress of various oxidative stresses leading to symptoms such as headache, nausea, anorexia, fatigue and lassitude. Regular consumption of *nak* cheese may also help to reduce the risk of oxidant-induced carcinogenesis.*Nak* milk and cheese has become an important ingredient of diet for the

people residing in higher altitudes to cope with oxidative stress brought about by direct UV exposure and rarefaction of the atmosphere and has helped them to stay healthy and live longer (Askew, 2002). The presence of antioxidants in higher amount in *nak* cheese might also be a possible explanation for itslonger shelf-life of over 4 month compared to cow cheese only ripened for a month, whereby higher concentration of antioxidants in nak cheese prevents considerable oxidative deterioration of the cheese.

5.7Influence of cheese components on probiotic bacteria

In this researchthere was a significant difference in OD at 550 nm between nutrient broth with isolates and cheese extract supplemented nutrient broth with isolates in conjugation with the different time interval of incubation ($P<0.05$) . It was demonstrated that cheese extract added to culture exerted influence on bacteria growth. The significant increase in OD of the broth with cheese extract was observed for 4 h compared to control. The increase in OD observed for 4 h culture ranged from 110- 144% of control to 190-256% for nutrient broth supplemented with cheese extract respectively. Extract of *nak* cheese (2900 m) with highestantioxidant activity, the increase in OD was highest indicating the positive influence of cheese components mainly the antioxidants on the viability of the bacteria present in it.The cheese extract is a mixture of antioxidants (phytochemicals & oligopeptides) and short chain fatty acids. The components in the extracts are transformed by the probiotic bacteria and utilized as energy source. The results are similar to previous research of Chodak, Tarko, and Statek on L. caesi which is representative of probiotic bacteria and Shabboo et al.,(2015) where he showed no antibacterial effects of phenolic compounds of *A. Sativum* on *S. thermophilus*.The interaction between phytochemicals and probiotic bacteria depend on the type of phytochemicals present and the strain of bacteria as explained by Tarko, (2008).

The components of the cheese may help to stabilize the probiotic bacteria in them during long period of ripening. The antioxidants neutralize the free radicals and the fatty acids can form a protective barrier around the LAB against the detrimental effects of free radicals as well as antibacterial peptides present in cheese, thereby creating a suitable environment for the viability of bacteria. It is suggestedthat not only antioxidants but various other unknown attributes of cheese affected the presence of probiotic bacteria in it. These attributes could be cheese matrix, water activity, moisture contents, possible interactions with other starter cultures and other microbial components of secondary cheese micro flora.

It should be understood that the interaction between antioxidants and bacterial strains included is not neutral. The interactions have to be taken into account whenever those components are present simultaneously in food products. On the one hand the antioxidants present in cheese may raise the nutritional and pro-health values (vitamins, antioxidants), but the antioxidant activity of plant antioxidants may be removed by bacterial transformation and the derivatives of different biological activity may be formed as a result. Similarly antioxidants in the cheese can also have detrimental effect on the bacterial

viability aswell. So selection of antioxidants and bacterial strains to be used in the cheese should be done wisely, as suggested by Tarko, (2008).

5.8 Brine shrimp lethality bioassay of cheese extract.

The brine shrimp lethality assay has been used routinely in the crude extracts of plant and food samples to assess the toxicity towards brine shrimp, which provides an indication of possible cytotoxic and anti-tumor properties of the test materials (Peteros and Yu, 2010). The variation in BSLA results may be due to the difference in the amount and kind of cytotoxic substances (e.g. flavonoids or phenols), the dietary proteins such as casein and whey and secondary microbial metabolites in the crude extracts of the cheese. As mentioned by Meyer and others, LC_{50} of less than 1000 µg/mL is toxic while LC_{50} of greater than 1000 µg/mL is non-toxic.According to the toxicity scale of Hodge and Sterner, LC_{50} value 0f 100-1000 is considered moderately toxic**.** Therefore considering scale given by both the researchers, the cheese studied are moderately cytotoxic**.**

Themoderate lethality of several cheese extracts to brine shrimp is an indicative of the presence of potent cytotoxic components and probably insecticidal compounds (Riser and Cortes, 1996) which may have effects on both normal and cancerous cells.Yasuda et al, 2013 reported lower cytotoxic effect of Pouligny Saint-Pierre cheese in differentiated HL-60 cells than undifferentiated HL-60 cells at the varying concentrations tested. According to a study conducted byYasuda et al.,(2010),highly ripened cow milk cheese demonstrated higher cytotoxicity through anti-proliferative activity, induction of apoptotic DNA fragmentation, and increase of nuclear morphological changes in HL-60 cells, possibly due to production of fermented and secondary metabolites by the microflora over long duration of ripening. But the toxicity of the studied cheese did not show any relation to their ripening duration in our experiment. The comparable cytotoxicity of cow cheese with one month ripening and low concentration ofcytotoxic substances (flavonoids or phenols), cannot be justified by the results of the research mentioned and possibly may indicate to presence of higher amount of dietary proteins (casein and lactoferrin) and microbial metabolites with cytotoxic potential.Based on the results of such experiments and the results of this study; consuming *nak* cheeseregularly in low dose might not have serious health implications but may exhibit anti-cancerous effects.

CHAPTER VI

CONCLUSIONS

Four types of Commercial cheese including one cow cheese and 3 *nak* cheese were sampled from cheese factories of Dairy Development Corporation located at different altitudes and regions of the country. Three plants foraged by *nak* were collected from the pasture and identified. The cheeses were subjected to total LAB count and total probiotic count. The cheese isolates were assayed for their probiotic properties and used to develop yoghurts which were comparatively studied with yoghurt prepared from BB-12. Phytochemical extraction using methanol as a solvent was carried out for the medicinal plants and cheeses. TPC and TFC of both plants and cheese extracts were determined. The cheeses were evaluated for their antioxidant activity through DPPH-radical scavenging assay and toxicity using brine shrimp lethality assay. Following conclusions can be drawn;

1. The functional aspects of *nak* cheese was studied by simple lab techniques. The sampled cheeses were evaluated for their probiotic potential. None of the selected cheese meet the criteriato have a functional food appeal in terms of presence of LAB with probiotic properties within the suggested minimal population of $>10^6$cfu/g for any probiotic containing product.
2. LABs meeting the criteria to be probiotics for use in GI tract were isolated from the sampled cheesewith probiotic properties. The isolated LABswere presumptively identified as galactose positive strains of *Streptococcus thermophilus*. Even though *lactobacillus* have widely been employed as probiotics with proclaimed health benefits none could be isolated from the selected cheese. Both cow cheese and *nak* cheese had predominant presence of *S. thermophilus*.
3. The isolated LABs were used to develop Yoghurt in the lab and comparative study was carried out with yoghurt prepared from commercial probiotic strain. The seven days viability of isolates obtained from cheese in the yoghurt was comparable to the commercial probiotic strain *Bifidobacterium animalis*imported from Denmark. The threehad comparable stability during seven days of refrigeration. Sensory attributes of yoghurt prepared from probiotic cheese isolates were comparable to yoghurt prepared from commercial strains. Therefore these strains may have industrial significance and represent an interesting candidate for use in biopreservation, probiotic food formulations and in control of food spoilage caused by food borne pathogens.

4. The residual phytochemicals in the cheese were comparatively very lower than in the plants foraged by Nak. The presence of phytochemicals and the antioxidant activity or moderate toxicitymight confer functional aspects to*nak* cheese a functional food and can be prescribed as a treatment to Oxidative stress through its regular consumption. Supported by the result of comparative analysis *nak* cheese of higher altitudes can be considered to have higher antioxidant activity.
5. Components of the studied cheese were able to exert significant influence on the viability of probiotic isolates.Proper understanding of interaction between probiotic bacteria and the antioxidant present in cheese is necessary.
6. The cheese extracts showed moderate cytotoxicity towards the brine shrimp used in bioassay, probably due to the presence of bioactive molecules such as phytochemicals or dietary proteinsin considerable amount, therefore necessitates assay of possible health effects of consuming *nak* cheese.
7. The results of this study supports the hypothesis that correlates the presence of pharmaceutically important phytochemicals in*nak* cheese with the feeding of medicinal plants of higher altitudes by the nak. Since phytochemical were present in significant amount in *nak* cheese, it can be said with confidence that medicinally important components of many herbs can pass on to the cheese via milk.*Nak*cheese produced in higher altitudes were found to have more antioxidant activities.
8. Present results showed that content of bioactive components are affected by the animals feeding system and geographical region. Outdoor grazing provided an increase of total polyphenol, and flavonoid concentrations in nak cheese compared to indoor fed cows. More research is needed to elucidate the potential of *nak* cheese as a functional food.

CHAPTER VII

SUGGESTIONS

After completing this research work following suggestions are forwarded to people who might be further interested in working on *nak* cheese or probiotics.

1. Although the research was focused on the commercially produced *nak* cheese of three different hilly regions, several indigenous nak cheese are produced throughout the Himalayan regions of the country. These indigenous cheeses should be studied for their functional aspects.
2. Dairy products and dairy industries are of great importance to the Nepalese people. Isolation of more potent and health beneficial probiotic microorganisms from indigenous dairy products and their effective delivery to the consumers through products should be done to contribute to better health of Nepalese people.
3. *Nak* cheese that has been an identity of Nepalese people should be promoted internationally for its health benefits. More in-depth study of this product should be carried out on various other aspects.
4. The probiotic strains isolated from *nak* cheese should be identified at species level and their novelty if established could replace the commercial strains imported from abroad. It necessitates the need of molecular identification of cheese isolates based on 16srRNA sequencing, since morphological characteristics and identical biochemical properties of LAB doesn't allow the accurate confirmation.
5. Moreover there is a necessity of identifying the phytochemicals present in the cheese more accurately. If the presence of pharmaceutically important plant components could be established in *nak* cheese, then various health benefits conferred by consuming them could be sincerely advocated beside the nutritional significance they hold.
6. The immunomodulatory and cytotoxic effects of*nak* cheese should be studied in detail so as to establish its correlation with occurrence or absence of any disease. Prevalence of allergenicity as well as long term health effects should also be identified before furthering any of its therapeutic values.

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APPENDIX

MRS Agar	**(Ingredients g/l)**	**Muller-Hinton agar**
Pepton 10.0 Lab-Lemcomeat extracts 10.0 Yeast Extract 5.0 D (-) Glucose 20.0 Tween 80 1ml $K_2HPO_4$2 Sodium acetate 5.0 Triammonium citrate 2.0 $MgSO_4.7H_2O$ 0.2 $MnSO4.4H_2O$0.05 Agar 15.0		30.0% beef infusion 1.75% casein hydrolysate 0.15% starch 1.7% agar

Table 8. 1: 9-point hedonic scale with scores and their corresponding labels.

Score	**Remarks**
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike Moderately
2	Dislike very much
1	Dislike extremely

Table 8. 2: Percentage increase in OD of the nutrient broth with isolates only and nutrient broth with isolates supplemented by extract of cheese at different time of incubation at 37^{o}C.

Time (hrs)	OD of nutrient broth with isolates(Control)				OD of nutrient broth with cheese extract and isolates			
					Cow cheese	Nak cheese		
	S-7	I-5	R-5	J-8	S-7	R-5	I-5	J-8
0	0.008	0.009	0.010	0.009	0.011	0.016	0.015	0.014
2	0.012	0.019	0.022	0.11	0.965	0.943	0.984	0.891
4	1.163	1.106	1.117	1.175	2.835	2.871	2.869	2.873
24	1.712	1.753	1.611	1.786	2.886	2.949	2.967	2.910

Each value is the average of the three absorbance at wavelength of 550 nm.Here isolates code; S-, R-, J- and P- stands isolates of *nak* cheese (2900 m), *nak* cheese (2400 m), nak cheese (2600 m) and cow cheese (1900 m) respectively.

Figure 8. 1: Total LAB count (left) and Probiotic Enumeration (right) by colony count on MRS media with 0.25% bile salts.

Figure 8. 2: Subculture of the Probiotic isolates on MRS agar at 37^{o}

Table 8. 3: Test for probiotic properties of the isolates (n=32) obtained from MRS media with 0.25% bile salts.

Isolates	Acid Tolerance test (3 h)			Bile concentration (4h)		Antimicrobial test (inhibition zone diameter in mm)		
	pH1	pH2	pH 3	0.3 %	0.25 %	*E. coli*	*S. aureus*	*Pseudomonas aeruginosa*
P-1	_	_	_					
P-2	_	_	_					
P-3	_	_	+	_	_			
p-4	_	_	_					
P-5	_	_	+	_	+	12	9	10
P-6	_	_	+	_	+	13	10	11
P-7	_	_	+	_	+	12	_	_
P-8	_	_	+	_	_			
J-1	_	_	+	_	+	11	10	9
J-2	_	_	_					
J-3	_	_	+	_	+	12	_	_
J-4	_	_	_					
J-5	_	_	+	_	+	13	_	_
J-6	_	_	_					
J-7	_	_	+	_	+	10	10	9
J-8	_	_	+	_	+	12	9	10
R-1	_	_	_					
R-2	_	_	+	_	+	13	11	10
R-3	_	_	_					
R-4	_	_	+	_	_			
R-5	_	+	+	_	+	13	10	12
R-6	_	_	+	_	+	9	7	11
R-7	_	_	+	_	+	10	8	11
R-8	_	_	+					
S-1	_	_	_					
S-2	_	_	+	_	_			
S-3	_	_	+	_	+	13	9	9
S-4	_	_	+	_	+	10	_	_
S-5	_	_	+	_	+	12	11	10
S-6	_	_	_					
S-7	_	_	+	_	+	14	12	9
S-8	_	_	+	_	+	12	_	_

(Where, blank cell indicates Exclusion from the test, + ve sign indicates positive test and _ sign negative test.

Here isolates code; S-, R-, J- and P- stands isolates of *nak* cheese (2900 m), *nak* cheese (2400 m), nak cheese (2600 m) and cow cheese (1900 m) respectively.

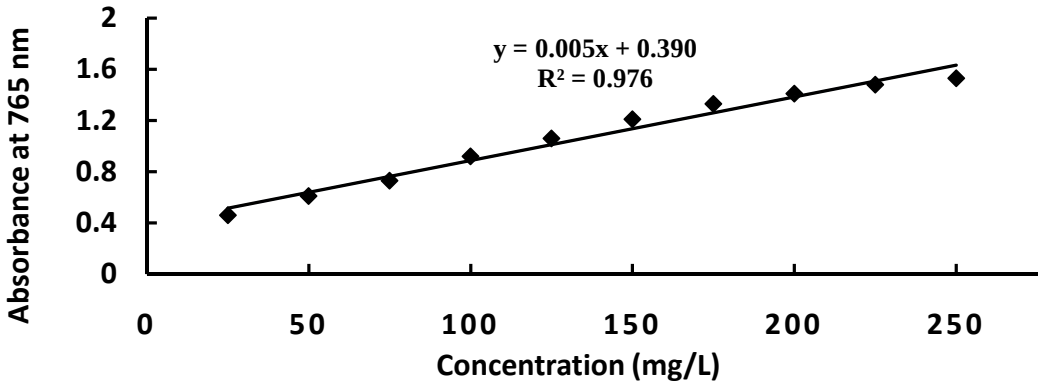


Figure 8. 3: Calibration curve of gallic acid at wavelength of 765 nm for total phenol determination.

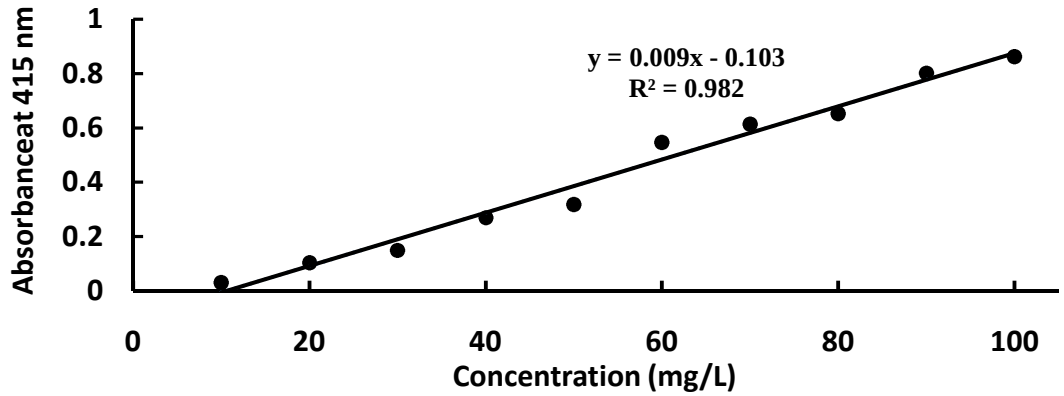
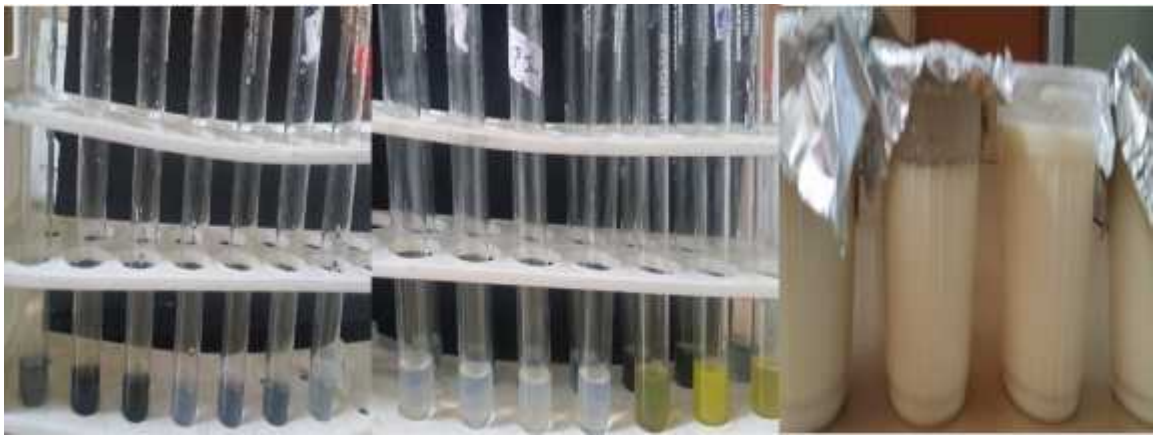


Figure 8. 4: Calibration curve of quercetin at wavelength of 415 nm for total flavonoid determination.

PHOTO GALLERY

A. Artemisia Vulgaris*B. *Rhododendron lepidotum* C. *Gaultheria nummularioides

D. NakE. Cheese ripening F. Phytochemical extraction



G. Phenol estimation H. Flavonoid estimation I. Lab prepared Yoghurt

J. Sensory evaluation of yoghurt