"Isolation and Characterization of Cholesterol Utilizing Probiotic Lactic Acid Bacteria"

Thesis Submitted for Master of Philosophy (M. Phil) Degree in Microbiology

of

Sikkim University

Kriti Ghatani

M.Phil 4th Semester

Regn No: 10SU2084

Roll No: 10PDMB02

2012

Department of Microbiology

Sikkim University, Tadong, 737102, Sikkim, India

सिक्किम विश्वविद्यालय
(भारतके संसदके अधिनियमद्वारा स्थापित केंन्द्रीय विश्वविद्यालय)
SIKKIM UNIVERSITY
[A Central University established by an Act of Parliament of India in 2007]

DECLARATION

I declare that thesis entitled **"Isolation and characterization of Cholesterol Utilizing Probiotic Lactic Acid Bacteria"** thesis submitted for the award of **Master of Philosophy (M.Phil)** Degree in **Microbiology** of **Sikkim University** is my original work. The content of this thesis is based on the experiments which I have performed myself. This thesis has not been submitted for any other degree to any oter university.

The content of this Mphil dissertation has been subjected to Anti Plagiarism Soft Ware (Ephorus) and it was found satisfactory.

Date : 30th June 2012.

Kriti Ghatani

Kriti Ghatani
Roll No.: 10PDMB02
Regn. No.: 10SU2084

We recommended that the thesis be placed before the examiners for evaluation.

Dr HK Tiwari
Coordinator
Department of Microbiology
Sikkim University

Dr BM Tamang
Supervisor

ASSISTANT PROFESSOR
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
6th Mile Samdur P.O. Tadong Sikkim

६ माइल, सामदुर, पीओ तादुङ–७३७१०२, गान्तोक, सिक्किम, भारत
फोनः00–९१–३५९२–२५१०६७, २५१४३८, फ्याक्स–२५१४३८
6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251438, Fax - 251438
website : www.sikkimuniversity.in

सिक्किम विश्वविद्यालय
(भारतके संसदके अधिनियमद्वारा स्थापित केंद्रीय विश्वविद्यालय)
SIKKIM UNIVERSITY
[A Central University established by an Act of Parliament of India in 2007]

CERTIFICATE

This is to certify that the thesis entitled **"Isolation and characterization of Cholesterol Utilizing Probiotic Lactic acid bacteria"** submitted to the **Sikkim University** for the award of **Master of Philosophy (M.Phil) Degree in Microbiology,** embodies the results of bonafide research work carried out by **Miss Kriti Ghatani** under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate ship and fellowship.

All the assistance and help received during the course of the investigation have been acknowledged by her.

Dr. BM Tamang
(Supervisor)

Gangtok, Sikkim
Date: 30 th June 2012

ASSISTANT PROFESSOR
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
6th Mile Samdur P.O. Tadong-Sikkim

ACKNOWLEDGEMENT

This thesis is my journey in obtaining my M Phil Degree. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well wishers, my friends and colleagues. At the end of my thesis I would like to thank all those people who made this thesis possible and an unforgettable experience for me. At the end of my thesis, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

At first I would like to express my genuine thanks to Dr. BM Tamang, my supervisor and teacher who gave me full support and encouragement to fulfil my research problem ardently. I am thankful for his detailed and constructive comments, and for his important support throughout this work under whose guidance.
I successfully overcame many difficulties and learned a lot.

I take this opportunity to acknowledge my sincere gratitude to Honourable founding Vice-Chancellor of Sikkim University Prof. Mahendra P. Lama, for his encouragement, support and providing us with the requisite infrastructure.

I wish to express my warm and sincere thanks to Professor J.P Tamang, Department of Microbiology Sikkim University, 6th Mile, Tadong, Gangtok who gave me important guidance during first steps into Food Microbiology.

I am thankful to Dr Nagendra Thakur, Assistant professor, Department of Microbiology, Sikkim University for providing valuable help and support, particularly for the screening of the cholesterol utilizing activity of the bacterial isolates. I am also thankful to Dr Bimala Singh, Assistant professor, Department of Microbiology, Sikkim University and Dr H K Tiwari, Associate Professor Department of Microbiology, Sikkim University for constructive criticism and excellent advice.

I take this opportunity to sincerely acknowledge the Rajiv Gandhi Fellowship Scheme (RGNF), Government of India, New Delhi, for providing financial assistance in the form of Junior Research Fellowship which buttressed me to perform my work comfortably.

I express my gratitude to Mr. Krishna Kant Yadav, my teacher during master's programme, University of North Bengal for his help, support, inspiration and blessings. I sincerely thank him for giving me valuable education during my post graduation.

I am thankful to Ms Samarmit Lepcha and her parents, student, Sikkim University for supplying me Yak milk samples from Lachung, North Sikkim. I also thank Ms Swastika Gurung student of Microbiology Sikkim University, for providing Yak churpi from Changu.

I am very thankful to Dr Manju Rana, Assistant professor, Department of Floriculture and Horticulture Management, Sikkim University for providing me the opportunity to let me work in the Horticulture Laboratory.

I am indebted to my many student colleagues Mr. Shankar Prasad Sha and Mrs Pramila Koirala for providing a stimulating and fun filled environment. My thanks go in particular to Mr. Shankar Prasad Sha who helped me a lot during my work. My special appreciation goes to my sister Smriti for her friendship and encouragement. Mr Gagan Chettri, Laboratory Assistant for his constant support and help in the Laboratory related work.

The university authority and Department of Microbiology have given me ample support while nourishing the ideas of creating thesis. It has provided us with Hi tech laboratory and all the necessary technical items and books.

I am also thankful to the members of the Food Microbiology Laboratory for providing me bacterial cultures of Lactic acid bacteria.I express my sincere thanks to Ms Niki Kharel, her selfless advice, help and support.

I express my sincere gratitude to my beloved parents Shri Krishnaraj Ghatani Smt.Tilanjali Singh, grandmother Smt Dilkumari Singh and all my family members for their love, support and help they have given me throughout a long run in my life and lifting me uphill this phase of life.

Date: 30th June 2012

Kriti Ghatani

Kriti Ghatani

CONTENTS

INTRODUCTION

Obesity and abnormal weight gains is a significant problem in several countries. It is estimated that there are 5 % affected persons in India. It is not a physiological disorder but the major cause of several diseases and it shows an increased risk of problems such as hypertension, cardiovascular disease, and certain type of cancers, diabetes and arthritis (American Academy of Paediatrics).

Cardiovascular disease (CVD), specifically coronary heart disease, stroke, congestive heart failure, and peripheral artery disease are the topmost cause of morbidity and mortality in industrialized countries in the twentieth century (Walker and Remy, 2009). Number of morbidity and mortality due to CVD is increasing in developing countries also due to urbanisation and decreasing use of traditional whole grains, fruits, vegetables, decrease in physical activity, and due to stressful life (Luepker, 2011).

Cholesterol is the main constituent of lipid found in blood, bile and brain tissues, it is required for the formation of sterols and cellular membranes. Source of cholesterol in the blood serum is either by synthesis in liver or the dietary cholesterol. Among dietary cholesterol, both the diet of plant origin and animal origin contributes build up of serum cholesterol. Absorbed cholesterol contributes increased risk of CVD (Assmann *et al.*, 2006; Matthan *et al.*, 2009). The link of CVD with elevated serum cholesterol and hypertension were established more than 50 years ago (Berkson *et al* 1960; Spilburg *et al* 2003). Hypercholesterolemia occurs when there is an elevated level of total cholesterol in the bloodstream. A small reduction in the serum cholesterol of 1% would reduce the risk of coronary heart disease by 2-3% (Frick *et al.*, 1987). In 2005, 53% of the deaths were on account of chronic diseases and 29% were due to CVD alone. It is estimated that by 2020, CVD will be the largest cause of disability and death in India (Reddy, 2005).

Source of cholesterol in the blood serum is either by synthesis in liver or the dietary cholesterol. Among dietary cholesterol, both the diet of plant origin and animal origin contributes build up of serum cholesterol. There are different types of cholesterol. Each kind has a different effect on the body. VLDL is produced in the liver and intestine and is responsible for the transport of indigenously synthesized triglycerol. LDL is formed from VLDL in the blood circulation. They transport cholesterol from other tissues. It blocks arteries which can lead to heart attack and

stroke. HDL is mostly synthesized in liver. Three fractions of HDL (Mann *et al.*, Bazarre *et al* .1983 and Hepner *et al*) can be identified by ultracentrifugation. HDL particles transport cholesterol from peripheral tissues to liver (reverse cholesterol transport) and keep arteries from blocked. Triglycerides are fats increase the chance of having a heart attack or stroke if the level in your blood is too high. Sometimes, our body makes more cholesterol than we really need and this excess cholesterol circulates in the blood stream. High levels of cholesterol in the blood can clog the blood vessels and increase the risk of heart attack and stroke. High concentrations of LDL promote atheroma development in arteries (arteriosclerosis). But high concentrations of HDL can remove cholesterol from cells and prevent atheroma formation. These balances are genetically determined and can be changed by body fluids, medications, food choices and other factors (Durrington, 2003). Many drugs like Lipitor, Crestor, Zocor, Pravachol, Lovastatin etc. have been found to possess ability to lower cholesterol level in humans but have side effects.

Absorbed cholesterol contributes increased risk of CVD (Assmann *et al.*, 2006; Matthan *et al.*, 2009). A small reduction in the serum cholesterol of 1% would reduce the risk of coronary heart disease by 2-3% (Frick *et al.*, 1987).

Lactic acid bacteria (LAB), particularly lactobacilli, streptococci and enterococci are important microbiota present in the human gastrointestinal (GI) tract (Marteau *et al.*, 2001; Haenel *et al.*, 1975). Beneficial role of these lactic acid bacteria present in the GI tract has already been reported by authors like Moro (1900), Cahn (1904) and Metchnikof (1907). These beneficial microflora mostly genera belonging to LAB and bifidobacteria found in the GI tract has now been increasingly used as probiotic micororganism. The word probiotics, literally meaning 'for life', live microorganisms, which when reaching the intestines in sufficient numbers (e.g., administered via food), will exert positive effects in humans and animals (Marteau *et al.*, 1995). Beneficial effects of probiotic bacteria are anti-mutagenic (Matar *et al.*, 1997; Bodana and Rao, 2011; Ambalam *et al.*, 2011) and anti-carcinogenic (Horinaka *et al.*, 2010; Kandasamy *et al.*, 2011), prevents gastric and intestinal disorders by preventing colonization by pathogenic and harmful microorganisms (Rani and Khetrapaul, 1998; Spanhaak *et al.*, 1998; Zubilaga *et al.*, 2001;

Apostolidis *et al.*, 2011), development of immune system (Wold, 1998; Holt, 1995; Cebra, 1999; Von Mutius, 2000), etc.

Since these probiotic lactic acid bacteria plays important role in fermentation process of many of the traditional food, microorganisms which has cholesterol lowering effect can be used to ferment such traditional food, targeting patients with hypercholesterolaemia. Since the early studies of Mann and Spoerry (Mann *et al.*, 1974) there has been an increasing interest in the cholesterol-lowering properties of fermented milk (FM) (yogurt) products, and numerous studies have focused on the potential hypocholesterolemic activity of FMs in humans. From these seven clinical studies, six reported a reduction in serum cholesterol concentration associated with FM intake.Traditional fermented foods are part of our normal diet; they will not have any side effects compared to the drugs prescribed for hypercholestrolaemia. Alternately, these probiotic microorganisms may be formulated in the form of capsules.

To the best of our knowledge no such study has been done till date on the cholesterol utilizing and bile acid degradation of LAB isolates from fermented milk products of yak and edible vegetable oil spill. The proposed dissertation work is aimed to study the ability of LAB isolated form fermented milk products of yak and vegetable oil spills, to utilize cholesterol and degrade bile acids. Those LAB isolates with potential cholesterol utilizing ability will be characterized for other probiotic property.

Objectives:

1. To isolate bacteria from fermented milk products of yak in Sikkim and vegetable oil spills of vegetable industries / packing industry of North Bengal Region.

2. To test the bacterial strains by presumptive tests of LAB.

3. To study and screen the isolated bacterial strains for cholesterol utilization.

4. To identify the strains having cholesterol lowering activity.

5. Characterization of probiotic property of cholesterol utilizing strains.

REVIEW OF LITERATURE

Lactic Acid Bacteria (LAB) are Gram-positive, catalase negative, non-sporeforming, non-respiring cocci or rods, microaerophilic and produce lactic acid when carbohydrates are metabolized (Axelsson, 1998; Klein *et al.*, 1998). They are associated with food derived from plant and animal origin as well in the large and small intestines of humans and animals (Heilig *et al.*, 2002). The first pure culture of a bacterium was "Bacterium lactis" (probably *Lactococcus lactis*), obtained by J. Lister in 1873 (Rogers *et al.*, 2006). Their beneficial association with the human gastrointestinal (GI) tract was shown hundred years ago by Moro (1900), Cahn (1904). At present about 20 genera of LAB has been recognized among which the principal ones are *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Axelson, 2004). *Bifidobacterium,* which are closely related to lactic acid bacteria in various physiological functions and in production of lactic acid but differs in percentage of guanine + cytosine (G + C %) content, have been placed in a different group of Actinomyceteceae (Buchanan *et al.*, 1974).

Beneficial effect of LAB has been advocated by the Metchnikoff (1907; 1908) based on the longevity of Caucasians due to high intake of fermented milk products. Along with lactobacilli, bifidobacteria were isolated from the feces of the breast fed infant (Tissier, 1905). Breast fed infants had lower incidence of intestinal upsets than compared to formula fed infants. Since then research on development of products containing live microorganisms, called probiotics that provides beneficial effect to the host is in progress (Holzapfel *et al.*, 1998). Probiotics are mono or mixed preparation of live microorganisms which when administered in humans and animals improve the normal indigenous flora of the intestine there by exerting beneficial effects (Havenaar *et al.*, 1992). Considering the generally regarded as safe (GRAS) status of LAB, they are good candidate for probiotic use (Salminen *et al.*, 1998). Their safety status has been assessed and reported by number of author (Aquirre and Collins, 1993; Gasser, 1994; Donohue and Salminen, 1996; Salminen and Donohue, 1996; Marteau and Salminen, 1997).

Researchers have shown link between probiotics and certain health conditions including ulceration, diarrhea, *Helicobacter pylori* infection, lactose intolerance, cancer, and immune system function (Zubilaga *et al.*, 2001). They reported that *L. acidophilus* and *B. bifidum* can prevent the colonization by *H. pylori* thus preventing gastritis and duodenitis, and that high intakes of fermented milk products were associated with reduced risk of ulceration. In the context of diarrhoea, it has been found that a fermented product containing *Lb. acidophilus* could inhibit growth of *Salmonella dysenteriae*, *Salmonella typhosa,* and *E. coli*. The beneficial effect has been attributed to antimicrobial substances produced by *L. acidophilus*, *in vivo*, which neutralize the enterotoxins of *E. coli* (Rani and Khetrapaul, 1998). *H. pylori* infection was found to be associated with the deficiency of *Lactobacillus* species in the stomach (Zubilaga *et al.*, 2001). More and more evidences from *in vitro* study has been found where *H. pylori* is inhibited by *Lb. salivarius* (Aiba , et *al.*, 1998; Bazhenov, et al., 1997; Kabir, *et al.*, 1997; Apostolidis, *et al.*, 2011). Modulation of composition and metabolic activities of indigenous microbiota of GI tract has been found in subjects consuming milk fermented with *Lb. casei* (Spanhaak, *et al.*, 1998). Probiotics have their implication for therapeutic use in inflammatory bowel disease. This disease includes the 2 major diseases, Crohn's disease and ulcerative colitis, and is a life-long and chronic inflammatory condition of the gastrointestinal tract, 6 probiotic bacteria *Lb. acidophilus, Lb. casei, Lb. salivarius, Lactococcus lactis, B. bifidum* and *B. infantis* has been used against pancreatitis that has reached the level of mortality (Besselink, *et al.*, 2008). Probiotic formulations have been shown to contain microorganisms that produce bacteriocin that inhibit the survival and growth of pathogenic organisms (Pelletier, 2001; Cotter, 2004). Nanomolar concentrations of bacteriocin called lantibiotics have been produced by Gram positive bacteria such as *Lc. lactis* that target muti-drug resistant microorganisms in the lipid 2 region of their cell walls (Pelletier, 2001; Lawton, 2007). *Lb. acidophilus and Lb. casei* complexes are most frequently used as probiotics (Klaenhammer and Kullen, 1999; Mercenier, *et al.*, 2003). Bacteriocin have ability to inhibit growth of closely related spoilage microorganisms and certain pathogenic microorganisms thereby has been shown to increase the stability if the food product during the storage and shelf life (Salminen, *et al.*, 1996). Thus, modulation of the gut flora and prevention of enteric infection with probiotics along with diet containing prebiotics and drugs may

ultimately provide the means to develop optimal gastrointestinal health in disease-prone individuals (DuPont and DuPont, 2011).

Yogurts prepared by inoculating *Lactobacillus delbruekii* ssp. *bulgaricus, S. thermophilus, L. acidophilus,* and *Bifidobacterium* are thought to be considered antimutagenic and anticarcinogenic (Pool-Zobel, *et al.*, 1993), and are potentially useful for inhibiting cancer (Desobry-Banon, *et al.*, 1999). Several other authors have also reported anticancer property of fermented milk in colorectal cancer (Saikali, *et al.*, 2004), reduction in tumor in rat models (Friend and Sahani, 1984), synthesis of anticancer peptide lunasin has been reported during sourdough fermentation by LAB (Rizzello, *et al.*, 2011).

LAB present in the human mucosal surface also improves the digestibility, absorption of mineral nutrition (Bergqvist *et al.*, 2005), degradation of anti-nutritive factors (Tamang, *et al.*, 2009) and biosynthesis of B vitamins (Turpin, *et al.*, 2010).Vendt, *et al.* (2006) and Saran *et al.* (2002) in independent studies have reported increase in food conversion ratio and nutrient digestibility in infants fed with probiotic supplement containing *Lb. rhamnosus* GG and *Lb. acidophilus* respectively. The huge enzymatic equipment of bacteria enables them to break down a wide range of nutritional constituents that cannot be metabolized by the host such as α-galacto oligosaccharides, inulin, resistant starches, and some anti-nutritional factors such as tannins or phytates responsible for the chelation of minerals including iron, zinc, magnesium and calcium (Hooper, *et al.*, 2002; Kurokawa, *et al.*, 2007). Finally they can synthesize vitamins, especially those of the B group such as riboflavin, folate, and cobalamin (Turpin, *et al.*, 2010). Gut microflora also influences bioavilability and effects polyphenols (Laparra and Sanz, 2010). The human microbiota facilitate the extraction of energy from food, provide accessory growth factors, promote post-natal terminal differentiation of mucosal structure and function, stimulate both the innate and adaptive immune systems, and provide 'colonization resistance' against pathogen invasion (Crowe, *et al.*, 1973; Rosebury, 1962; Mackowiak, 1982; Smith *et al.*, 2007).

Probiotics also play a major role in boosting the stimulation of the host immune system by various specific and non-specific pathways (Klaenhammer, 2008) which involves the modification and regulation of humoral, cellular, and nonspecific

immunity (Gill, 2001). Some papers have reported amplified mucus production, macrophage activation, and stimulation of secretory IgA, decreased proinflammatory cytokine production, and increased peripheral immunoglobulin production by lactic acid bacteria (Villena, 2008). *Lactobacillus acidophilus* (Schiffrin, 1994) and *Lactobacillus rhamnosus* (Pelto *et al.*, 1998) have been reported to cause the enhancement of the level of phagocytosis. *Lb. acidophilus* strain and *Lb. rhamnosus* strain have shown to stimulate phagocytosis in allergic patients (Pelto *et al.*, 1998).

Plant sterols are important in agricultural products related to health and nutrition. Soysterols, vegetable oil components and sitosterol having hypocholesteromic acitivity have been documented (Jones *et al.*, 1998, Miettinen *et al* 1995). Sterols also decrease the absorbtion of fat soluble vitamins (Katan *et al.*, 2003). They help in the reduction of cholesterol by competing with the dietary and biliary cholesterol for the uptake into micelles (Melnikov *et al.*, 2004). The prescence of specific transporters facilitate the movement of bile acid micellies to the border of enterocytes (Kramer *et al.*, 2000). This mechanism of cholesterol transport has been exploited on the basis of use of a tracer called 2-azetidinones which lowers cholesterol (van Heek *et al.*, 1997). The saturated analogues of phytosterols and their esters have found an important use in lowering cholesterol levels in humans providing cardiological benefits (John *et al.*, 2007). Phytosterols are found in a number of Brassicaceae species that include *Brassica napus, B.* campestris, *B.juncea, B. nigra, Sinapis alba* and S. *arvensis* (*Brassica kaber*). S. *arvensis* is a wild type of mustard and records have shown that there is 2% cholesterol in the sterols of these plant. The chemical structure of plant sterols, campesterol and sitosterol, are very similar to that of cholesterol, they only differ with respect to the degree of absorption (Patel *et al.*, 2006; John *et al.*, 2007).

The isolation and characterization of sterols have been carried out using various techniques like column chromatography (CC), gas chromatography (GC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), reversed-phase HPLC and capillary electro chromatography (CEC).Sterols can also be detected by flame ionization detection (FID), UV detection (UV), evaporative light scattering detection (ELSD), infrared detection (IR), nuclear magnetic resonance detection (NMR) and mass spectrometry (MS) (Abidi, 2011).

Yaks belong to the cattle family and can live on only at high altitude above 10,000ft living on alpine undergrowth and can handle without food for many days and can survive in snow because of their thick hair. In Sikkim, Yaks are found above Lachung or Lachen, they have been domesticated by local people for consumption of meat and their milk products.Yak milk is rich in fat and solids comprising around 18 percent of solids and includes about 7 percent of fat. The milk has a aromatic, fairly sweet smell and whole milk also tastes somewhat sweet, without addition of sugar. A hardened cheese called churpi made from yak milk is very well-known in Sikkim. Churpi is prepared from buttermilk. The buttermilk is boiled and the solid mass obtained is separated from the liquid and wrapped and hung in a thin cloth to drain out the water. To prepare the hard variety, the soft mass is wrapped in a jute bag and pressed hard to get rid of the water. After it dries, it is cut into small cuboidal pieces and hung over fire to harden it further.

Recently, researches have also shown that probiotic microorganisms reduces cholesterol levels in several experimental animals (Mann and Spoerry, 1974; Rao *et al.*, 1981; Grunewald, 1982; Gilliland *et al.*, 1985; Danielson *et al.*, 1989; Lin *et al.*, 1989, 1995; Fukushima and Nakano 1996; James, 1999). Hosono (2000) reported inhibition of re-absorption of conjugated bile acids and increased removal of cholesterol from blood through feces by *Lb. acidophilus* in hypercholesterolemic rats. Similar study in mice with lactobacilli lowered total blood cholesterol by 22% and triglycerides by 33% (Taranto, 1999). Agerholm-Larsen (2000) reported lowering of blood pressure by diet supplemented with lactobacilli while the control group without lactobacilli did not show any reduction in blood pressure. Food cholesterol ingested is absorbed in the small intestine and some of the cholesterols are conjugated to glycine, taurine, bile salts, phospholipids to form micelle (Araki et al., 1996). While bile acids secreted into the intestine are normally deconjugated by bile salt hydrolase of lactic acid bacteria (Corzo *et al.*, 1999). These free bile salts being less soluble cannot be absorbed by the intestine resulting in lowering of cholesterol level (Liong *et al.*, 2005; Sugano, 1986) though this hypothesis has been disputed by some authors (Marteau *et al.*, 1995). Since high blood cholesterol leads to coronary heart diseases, prevention of cholesterol absorption in intestine may prevent exogenous source of cholesterol (Cheeke, 2000).

Rationale and Scope of the Study

Cardiovascular disease (CVD), specifically coronary heart disease, stroke, congestive heart failure, and peripheral artery disease are the topmost cause of morbidity and mortality in industrialized countries in the twentieth century (Walker and Reamy, 2009). Number of morbidity and mortality due to CVD is increasing in developing countries also due to urbanisation and decreasing use of traditional cultures based on grains, fruits, vegetables, heavy physical activity, and due to stressful life (Luepker, 2011). In 2005, 53% of the deaths were on account of chronic diseases and 29% were due to CVD alone. It is estimated that by 2020, CVD will be the largest cause of disability and death in India (Reddy, 2005).

Cholesterol is the cause of coronary heart disease. Since source of cholesterol for humans is both the dietary cholesterol as well as by biosynthesis in the liver, if the dietary cholesterol can be prevented from re-absorption then level of cholesterol in the blood can be reduced to a great extent. The aim of the study is to isolate a probiotic lactic acid bacterium from fermented milk products of yak and from oil spills of a vegetable oil industry. Since there is already reports on inhibition of cholesterol absorption by probiotic bacteria in various *in vivo* and animal models, LAB isolates from fermented milk products of yak and vegetable oil industry will be screened for cholesterol utilization and deconjugation of bile acids. To the best of our knowledge the LAB isolated from fermented yak milk products of Sikkim has never been screened for cholesterol reduction property. LAB isolates from vegetable oil industry or packing site has also not been screened for cholesterol utilisation activity. Bacteria especially lactic acid bacteria have been documented for beneficial health promoting effects earlier. The Cholesterol lowering properties of some Lactic acid bacteria had also been reported. The isolation of the beneficial bacteria with reference to cholesterol lowering properties had been documented by Mann and Spoerry (Mann *et al.*, 1974).There has been an increasing interest in the cholesterol-lowering properties of fermented milk (yogurt) products, and numerous studies have focused on the potential hypocholesterolemic activity of fermented milk in humans.

Since, the isolation of bacteria from Yak milk and fermented Yak milk products of Sikkim have not been studied till date with respect to the cholesterol lowering properties so the study was mainly based on the isolation of such

bacteria. Yak milk is rich in fat and solids, so there might be possibility that some cholesterol degrading bacteria might be present in Yak milk source.

The other Sample source is the vegetable oil spill sample from North Bengal region; plants contain sterol that is similar to cholesterol. Soysterols, vegetable oil components and sitosterol having hypocholesteromic acitivity have been documented (Jones *et al.*, 1998, Miettinen *et al* 1995). The sitosterol in plants have been reported to lower cholesterol levels as well, hence we assume cholesterol degrading bacteria to be present in this source as well. Sterols known as phytosterols are found in a number of Brassicaceae species that include *Brassica napus, B.* campestris, *B.juncea, B. nigra, Sinapis alba* and S. *arvensis* (*Brassica kaber*). A wild type of mustard, *Sinapis arvensis* has shown that there is 2% cholesterol in the sterols of this plant. The chemical structure of plant sterols, campesterol and sitosterol, are very similar to that of cholesterol, they only differ with respect to the degree of absorption (Patel *et al.,* 2006; John *et al.,* 2007).

Bile utilizing or bile acid deconjugating isolates will be further tested for other probiotic property such as tolerance to acidic pH and bile salt, hydrophobicity of cell surface, inability to produce biogenic amines, utilization of oligosaccharides and production of bacteriocins. Those LAB isolates with all of these properties or with most of these properties can be considered as the suitable strain for probiotic use along with ability to reduce cholesterol.

Once the cholesterol reducing bacterial isolates will be characterized, future studies can be conducted using radioactive cholesterol to find out the mechanisms of cholesterol absorption or degradation by probiotic bacteria. *In vitro* studies and rat model studies can authenticate the cholesterol reduction hypothesis of probiotic microorganisms to a great precision. In future, such cholesterol lowering probiotic lactic acid bacteria can be used either in the form of live bacteria or as starter culture in fermented food by hypercholesteromic patients(Gibson *etal.*,2004).However dairy products are considered as ideal carriers of probiotics for the delivery of its action (Rasic, 2003).

METHODS AND MATERIAL

3. Materials and methods

3.1. Culture media used

(1). Arginine Hydrolysis Medium (Thornley, 1960)

Peptone	10.0 g
Yeast extract	5.0 g
D (+) glucose	0.5 g
$K_2HPO_4.3H_2O$	2.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Sodium acetate	5.0 g
Tri-sodium citrate	20.0 g
Tween 80	1.0 ml
Arginine	0.3 %
Phenol red	0.01 g
Distilled water	1000 ml
pH	5.0

(2). Bacteriocin Screening Medium (Tichaczek *et al.*, 1992)

Peptone	10 g
Beef extract	5.0 g
Yeast extract	5.0 g
Glucose	2.0 g
K_2HPO_4	2.0 g
Tween 80	1.0 g

Diammonium citrate	2.0 g
Sodium acetate	5.0 g
$MgSO_4$	0.1 g
$MnSO_4$	0.05 g
Distilled water	1000 ml
pH	6.5
Agar	12 g

(3). Biogenic Amine Sub-culturing Medium (Bover-Cid and Holzapfel, 1999)

MRS Broth (M369, HiMedia, Mumbai)	52.2 g
D-Tyrosine (RM 1520, HiMedia, Mumbai)	1.0 g
L-Histidine monohydrochloride (Merck)	1.0 g
L-Lysine monohydrochloride (Merck)	1.0 g
L-Ornithine monohydrochloride (Merck)	1.0 g
Pyridoxal-5-Phosphate (RM 1554, HiMedia)	0.001 g
Distilled water	1000 ml
pH	6.00

(4). Biogenic Amine Screening Medium (Joosten and Northold, 1989; modified by Bover-Cid and Holzapfel, 1999)

Tryptone	5.0 g
Yeast extract	5.0 g
Meat extract	5.0 g
Sodium chloride	2.5 g
Glucose	0.5 g

Tween 80	1.0 g
K_2HPO_4	2.0 g
Ammonium citrate	2.0 g
Calcium carbonate	0.1 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.05 g
$FeSO_4.7H_2O$	0.04 g
Thiamine	0.001 g
Pyridoxal-5-phosphate	0.005 g
Bromocresol purple	0.05 g
Agar	22.0 g
Amino acid	5.0 g
Distilled water	1000 ml

Amino acids used were D-Tyrosine (pH 5.3) (RM 1520, HiMedia, Mumbai); L-Histidine monohydrochloride (pH 5.0) (Merck, Germany); L-Lysine monohydrochloride (pH 5.15) (Merck, Germany); L- Ornithine monohydrochloride (pH 5.0) (Merck, Germany).

(5) MRS Agar

Peptone	1g
Beef extract	0.8g
Yeast extract	0.4g
Glucose	2g

Sodium Acetate	0.5g
Tween 80	0.1g
Di potassium di hydrogen phosphate	0.2g
Magnesium sulphate	0.02g
Manganese sulphate	0.005g
Agar	2g
Distilled water	100ml
Ph	6.2 at 25 ^{0}C

(6). MRS Broth – Composition similar to MRS agar but broth does not contain agar.

(7). Medium A

Ammonium nitrate	17g
Di Potassium Hydrogen Phosphate	0.25g
Magnesium Sulphate	0.25g
Iron Sulphate	0.001g
Sodium chloride	0.005g
Tween 20	0.1ml
Cholesterol A R	3gm
Distilled water	1000ml

Cholesterol is first dissolved in 10ml solution of 20% butanol and 10% Tween 20 then filter sterilized through cellulose acetate membrane and added to the medium.

(8). Nutrient Agar

(9). Nutrient Broth (M002, HiMedia, Mumbai)

3.2. Reagents

(1). Acidic Ninhydrin

1-Butanol/water saturated	465 ml
Acetic acid	35 ml
Ninhydrin	2.5 ml

(2). Autospan Liquid Gold Span Diagnostics, Cholesterol Kit

(CHOD- PAP KIT)(Cognet)

(3). Gram's Crystal Violet (S012, Hi Media, Mumbai)

(4). Hydrogen peroxide

(5). Potassium Hydoxide

(6). Nessler's Reagent

Potassium iodide	50.0 g
Mercuric chloride (saturated)	35.0 ml
Distilled water (ammonia free)	25.0 ml
Potassium hydroxide (50 %)	400.0 ml

Potassium iodide was dissolved in 35 ml of distilled water followed by addition of saturated aqueous solution of mercuric chloride till the appearance of precipitate. Then, 400 ml of potassium hydroxide was added and made the final volume to 1000 ml by adding distilled water. The solution was left for a week; the supernatant was decanted and stored in capped amber bottle at 4° C.

(7). Ringer solution (Merck, Germany)

(8). Safranin (S027, Hi Media, Mumbai)

3.3. Reference Strains

Reference Strains	Origin	Purpose in this experiment
E coli	SBK	Indicator strain for antimicrobial activity
Lactobacillus plantarum Ek4	FMR	Indicator strain for antimicrobial activity
Lactobacillus casei Ek 35	FMR	Indicator strain for antimicrobial activity
Pseudomonas aeruginosa 1034	MTCC	Indicator strain for antimicrobial activity
Pediococcus pentosaceus R1B1	FMR	Indicator strain for antimicrobial activity
Proteus vulgaris 742	MTCC	Indicator strain for antimicrobial activity
Bacillus subtilis	SBK	Indicator strain for antimicrobial activity

Originally, these reference strains were obtained from, MTCC (Microbial Type Culture Collection, Chandigarh, India), SBK(Subashree Biotech, Kolkata) and FMR (Food Microbiology Laboratory, Sikkim Government College, Gangtok, India).

Staphylococcus aureus 7443, *Klebsiella pneumonia* 3384, *Pseudomonas aeruginosa* 1034 were propagated in standard nutrient agar. *Lactobacillus plantarum* Ek 4, *Lactobacillus casei* Ek 35, was cultivated in MRS broth. The cultures were maintained as frozen stocks at –80° C in 15% glycerol.

3.4. Collection of samples

Two yak milk samples and two fermented milk product (locally called *churpi*) of yak from Lachung and one from Changu, North Sikkim were collected. Three oil spill samples from vegetable oil industry or packing industry in Paribhanagar, three from Matigara and two samples from Jalpaiguri were collected. A sum of five Yak milk samples from Lachen region of North Sikkim and eight samples from North Bengal region were collected. All samples were collected aseptically in sterile bottles and poly-bags, which were kept in an ice-box container, and transported to the laboratory for analyses.

3.5. Microbiological analysis

Ten g of the oil spill sample were homogenised with 90 ml of 0.85 % (w/v) sterile physiological saline in a mortar and pestle. A serial dilution (10^{-1} to 10^{-8}) in the same diluent was made. One ml of the Yak milk sample was added to 9ml of 0.85 % (w/v) sterile physiological saline and diluted. Since *Chupi* samples were very hard and difficult to break, it was immersed in 20 ml of 0.85 % (w/v) sterile physiological saline for 24-48 hrs followed by dilution. Lactic acid bacteria (LAB) were isolated on MRS agar (M641, HiMedia, Mumbai) supplemented with 1 % $CaCO_3$, and incubated under anaerobic condition in an Anaerobic Gas-Pack system (LE002, HiMedia, Mumbai) and incubated at 30° C for 48-72 hour. Aerobic mesophilic counts were determined using plate count agar (M091A, HiMedia, Mumbai) incubated at 30° C for 48-72 hour. Colonies were selected randomly or all sampled if the plate contained less than 10 colonies, according to Leisner *et al.* (1997). Purity of the isolates was checked by streaking again on fresh agar plates of the isolation media and sub-culturing on corresponding broths/agar, followed by microscopic examinations. Microbiological data obtained were transformed into logarithms of the numbers of colony forming unit (cfu) per g of sample. All the strains of bacteria were preserved in MRS broth using 30 % (v/v) glycerol at –80° C.

3.6. Preliminary Characterisation of Bacterial Isolates

3.6.1. Cell morphology

Smear of a 24 hour-old bacterial culture was made in a grease free slide, air-dried (not heated-fixed), it was stained for 30 sec with safranin (S027, HiMedia, Mumbai), washed in water, air-dried (Harrigan, 1998) and observed under oil-immersion objective. Cell dimensions were measured with a standardized ocular micrometer.

3.6.2. Production of catalase

The production of gas bubbles by the isolates were observed by adding 0.5 ml of 10 % hydrogen peroxide solution (Merck) to the drop of cultures that were grown in broth overnight indicating the presence of catalase (Schillinger and Lücke, 1987).

3.6.3. Ammonia from arginine

5 ml arginine hydrolysis medium in tubes (Thornley, 1960) were inoculated with 24 hour-old culture. The tubes were incubated at 30° C for 3 days and formation of ammonia from arginine was detected by spotting 100µl culture onto a white porcelain tile and adding equal volume of Nessler's reagent. Appearance of dark orange colour indicated presence of ammonia. (Schillinger and Lücke,1987).

3.6.4. Gas ($C0_2$) production from glucose

MRS broth tubes of 10 ml, without citrate and containing inverted Durham tubes was inoculated with 24 hour-old cultures and incubated at 30° C (Schillinger and Lücke, 1987). Accumulation of gas in the inverted Durham tubes indicated positive result.

3.6.5. Growth at different pH

The pH of MRS broth was adjusted to 3.9 and 9.6 using 1 N HCl or 10% w/v NaOH. The medium was distributed into tubes containing 5 ml in each. They were autoclaved, cooled to room temperature and inoculated with 24 hour-old MRS broth culture. The tubes were incubated at 30° C for 24-72 hour and observed for growth (Dykes *et al.*, 1994).

3.6.6. Growth at different temperatures

MRS broth were inoculated with 24 hour-old cultures and incubated at 10° C and 15° C for 7 days, and 45° C for 3 days, respectively and observed for growth (Dykes *et al.*, 1994).

3.6.7. Growth in different NaCl concentrations

Tolerance to salt was tested by inoculating a loop-full of culture in MRS broth supplemented with 6.5 %, 10.0 % and 18.0 % NaCl, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987). Cultures were observed for growth after incubation for 72 hrs.

3.6.8. Acid from carbohydrates

The method was based on Schillinger and Lücke (1987). Tubes of 5 ml MRS broth without beef extract, containing 0.5% w/v of six different carbohydrates instead of glucose and 0.004% phenol red indicator were inoculated and incubated at 30° C for 2-5 days. Colour change from red to yellow indicated acid production.

3.6.9. Phenotypic identification

Bacterial species were identified following the taxonomic keys of Bergey's Manual (Sneath *et al.*, 1986), and Wood and Holzapfel (1995). Only those strains showing cholesterol reducing property were identified using Biolog Microbial Identification System.

4. Probiotic Properties of LAB Isolates

4.1. Screening of the identified bacteria for Cholesterol lowering effect:

The isolated strains can be cultured in medium A (Nagasawa *et al.*, 1969) was modified by including amino acids in broth medium. The medium is supplemented with 2% cholesterol which is the only carbon source available. Cholesterol was dissolved in 10 ml of 20% butanol and 10% tween 20, filter sterilized through a membrane filter of 0.45µm pore size. The cholesterol solution was added to the broth medium and distributed in 5ml each in duplicates. 10µl of the test organism was inoculated and incubated at 30°C for 7 to 12 days. Control tubes were not inoculated with any bacterial culture. The concentration of total cholesterol utilization was quantitatively determined *in vitro* utilizing the cholesterol estimation kit (Span Diagnostics Ltd.).

The principle behind the total cholesterol determination method is based on CHOD-PAP method. Cholesterol esters are hydrolysed by Cholesterol esterase (CE) to give free Cholesterol and Fatty acids. In subsequent reaction, Cholesterol oxidase (CHOD) oxidises the 3-OH group of free Cholesterol to liberate Cholest-4-en-3-one and Hydrogen Peroxide couples with 4-Aminoantipyrine (4-AAP) and Phenol to produce Red Quinoneimine dye. Absorbance of the coloured dye is measured at 505nm and is proportional to amount of Total Cholesterol concentration in the sample.

Those strains which showed cholesterol utilizing ablitity were further identified by Biolog Microbial Identification System.

4.2. Identification

Biolog Identification system

The Biolog microplate bacterial identification system (Biolog Inc., USA; Oxoid GmbH, Wesel, Germany) based on the utilisation of 95 single carbon sources. The metabolism of the substrates in the wells of the microplates results in a reduction of tetrazolium dye producing a colour change, and a specific "metabolic fingerprint'' was obtained for each strain and compared with the data of the Biolog MicroLog database software (Biolog Inc.). Before inoculation of the Biolog AN microplates, strains were grown anaerobically on MRS agar at 30° C for 48 hour. The bacterial cells were streaked from the surface of the agar to generate well isolated colonies and suspended in Inoculating fluid (Biolog Inc.). The top of the colony should be touched lightly with the inoculators swab provided. The swab is then inserted into the inoculating fluid and the inoculating fluid tube is shaken vigorously. The cell density transmittance was measured using turbidometer with a transmittance range of 90-98%. The specified amount of cell suspension is pipetted into each labelled MicroPlate, the lid put on, and placed in trays in incubator.

MicroPlates are incubated at 30°C. After an appropriate incubation time, OmniLog Identification System automatically reads each MicroPlate. The patterns formed in the wells are automatically entered into OmniLog Identification System, which searches the database and can provide an identification call in seconds.

5. Characterization of the identified bacteria for probiotic properties

5.1. Acid Tolerance

The identified strains were grown in MRS broth for 6 hrs at 37 °C. 10µl of the 6 hr old culture was inoculated to 5 ml of the respective tubes containing MRS broth whose pH was adjusted to 3 and 7 using 1N HCl and 1N NaOH respectively. Bacterial growth was monitored by determining the optical density at 620 nm after 24 hrs incubation period at 37°C. The difference in percentage between the variation of optical density (OD) at a pH 7 (OD pH 7) and variation of optical density (OD) at a pH 3 (OD pH 3) give the index of the isolates surviving that can be expressed as follows:

$$\text{Surviving Percentage \%} = [(\text{OD pH7} - \text{OD pH3}) / \text{OD pH7}] 100$$

Classification criterion included a random level of acid condition tolerance: good if the isolate survived at pH 3 after 24h and poor if the isolate did not survive in any experimental condition. An isolate survived if it demonstrated a surviving percentage equal or greater than 50 %. (Pelinescu *et al.*, 2009)

5.2. Bile Tolerance activity

The identified strains were grown in MRS broth for 6 hrs at 37°C. 10µl of the 6 hr old culture was inoculated to 5 ml of the respective tubes containing MRS Broth supplemented with 0.4% bile salt using a modified method described by Dora and Glenn (2002). Bacterial growth was monitored by determining the optical density at 650 nm after 24 hrs incubation period at 37°C. The difference in percentage between the variation of optical density (OD) at 0% BS (OD 0%BS) and variation of optical density (OD) at a 0.4% BS (OD 0.4% BS) give the index of the isolates surviving that can be expressed as follows:

$$\text{Surviving Percentage\%} = [(\text{OD 0\%BS} - \text{OD 0.4\% BS}) / \text{OD 0\%BS}] 100$$

Classification criterion included a random level of bile condition tolerance: good if the isolate survived at 0.4% bile salt after 24h and poor if the isolate did not survive in any experimental condition. An isolate survived if it demonstrated a surviving percentage equal or greater than 50 %.(Pelinescu *et al.* 2009)

5.3. Hydrophobicity assay

Bacterial adhesion to hydrocarbons was determined and results were expressed according to Rosenberg (1984) and Perez *et al.* (1998), modified as follows. Fresh cultures were grown in MRS broth at 30° C for 24 hour and centrifuged at 8,000 *g* for 5 min. The pellet was washed three times with 9 ml of Ringer solution (Merck, Germany), and thoroughly mixed in a vortex. The 1 ml of the suspension was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of the suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia, and Mumbai) in duplicates and mixed thoroughly in a vortex. The phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully removed and absorbance at 580 nm was measured. The percentage hydrophobicity was expressed as follows: hydrophobicity % = $[A_0\text{-}A/A] \times 100$, where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane. The percent hydrophobic index greater than 70 % was arbitrarily classified as hydrophobic (Martin *et al.*, 1989; Nostro *et al.*, 2004).

5.4. Biogenic amine Production

The ability of LAB isolates to produce biogenic amines was determined qualitatively on an improved screening medium as described by Bover-Cid and Holzapfel (1999) using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Cultures previously grown and sub cultured twice in biogenic amine sub-culturing medium were spotted onto the plates containing screening medium. Change of the bromocresol purple indicator to purple was considered as index of significant amino acid decarboxylase activity, corresponding to >350 mg of a particular amino acid/litre (Olasupo et al., 2001).

5.5. Antimicrobial activity

The LAB isolates were screened for antimicrobial activity by agar spot method of Schillinger and Lücke (1989). The indicator strains used for antagonisms included: *Staphylococcus aureus* 7443, *Klebsiella pneumonia* 3384, and *Pseudomonas aeruginosa* 1034, *Bacillus cereus* FMR, *E coli* (Subashree Biotech, Kolkata) and *Proteus vulgaris* 742. The LAB isolates were screened for bacteriocin production by agar spot test method described by Uhlman *et al.* (1992), using the bacteriocin screening medium of Tichaczek *et al.* (1992).

How much sample spotted ?

RESULT

6.1. Microbial Population

Two different sample sources were considered for the study. Eight different types of mustard oil spill samples were collected from different places in North Bengal region that included Paribhan Nagar (3), Matigara (3) and Jalpaiguri district (2). Six Yak milk and its product, Churpi were collected from the North region of Sikkim which included Lachen (2), Lachung (2) and Changu (1).In all the Oil Spill samples the population of LAB as well as other aerobes were in the range of 10^6 to 10^8 cfu /ml. The count of LAB in Yak milk sample was less being 10^5 cfu/ml. Total viable counts (TVC) was additional in the Yak churpi sample as compared to the Yak milk sample.

Table 1: Microbial population of oil spill sample Collected from different parts of North Bengal .

Product	Place of collection	Log Cfu/g	
		TVC	Bacteria on MRS agar
Mustard Oil spill	Paribhan Nagar (n= 3)	8.1±0.06	6.7±0.07
	Matigara (n= 3)	8.5±0.07	6.9±0.09
	Jalpaiguri (n= 2)	7.2±0.08	6.6±0.07

n= number of samples. Data represents the mean (±SD) of three sets of experiment samples. LAB, lactic acid bacteria; TVC, Total viable count

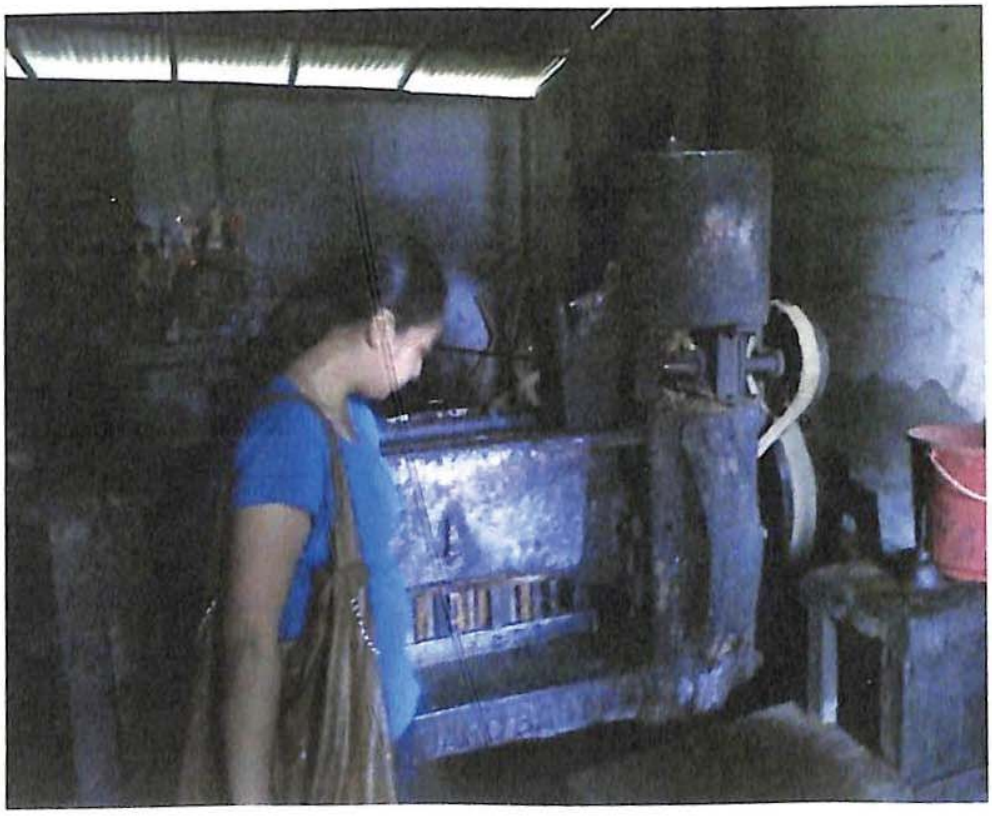
Table 2: Microbial population of Yak Milk and Yak Churpi sample collected from different part of North Sikkim.

Product	place of collection	Log cfu/g	
		TVC	Lactic Acid Bacteria
Yak Milk	Lachen (n= 2)	7.4±0.07	6.5±0.07
Yak Churpi sample	Lachung (n= 2)	8.1±0.07	6.9±0.08
	Tsongmo Lake or Changu Lake (n=1)	8.2±0.09	6.8±0.09

n= number of samples. Data represents the mean (±SD) of three sets of experiments.

LAB, lactic acid bacteria; TVC, Total viable count

(a)



(b)

Photograph .1. (a).and (b).Sample collection at a small scale industry in Paribhan Nagar, Matigara, North Bengal.

Photograph 1. (c). Sample bottle containing Oil spill Sample, Paribhan Nagar, Matigara, North Bengal.

(b) (a)

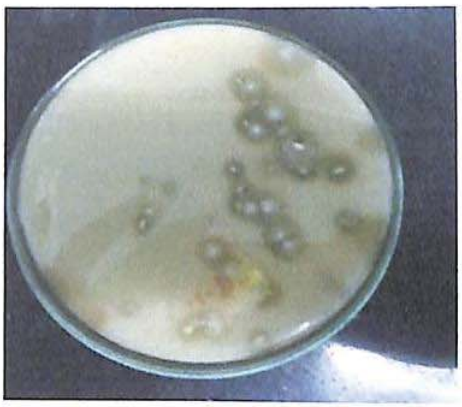
(c)

Photograph 2. (a).An image of Yak from the Changu region,Sikkim.

Photograph 2. (b).Sample bottle containing Yak Milk Sample,Lachen ,North Sikkim.

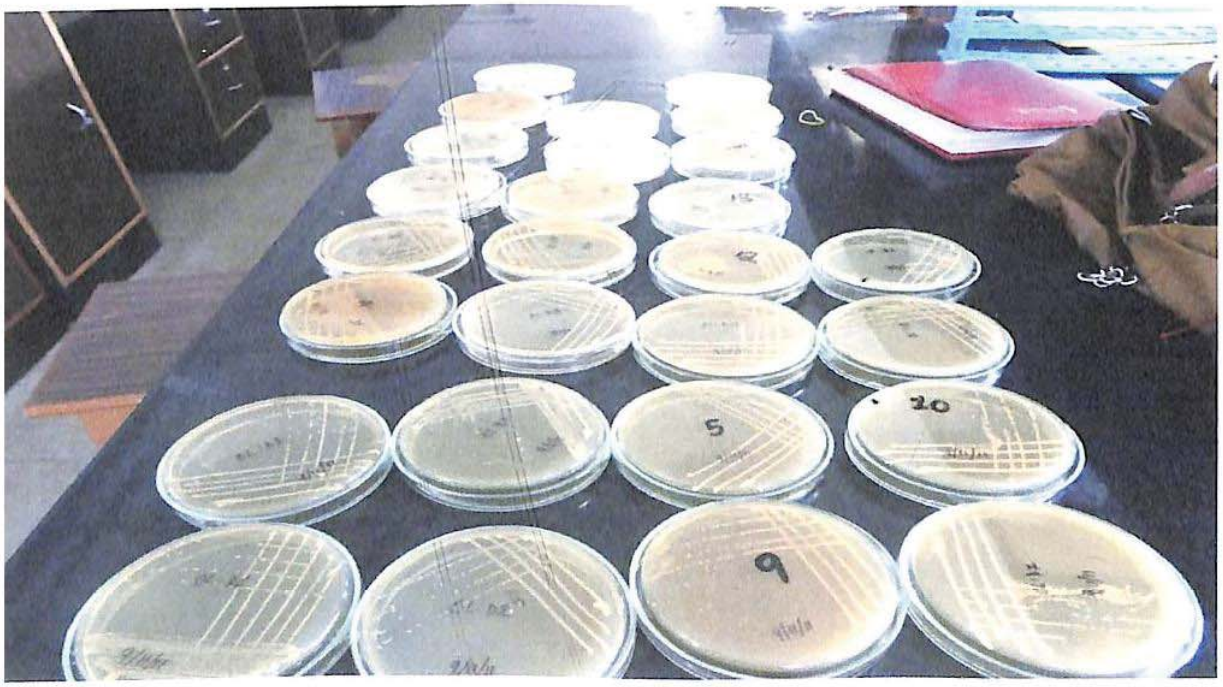
Photograph 2. (c).Churpi,a fermented Yak milk product,Lachun,.North Sikkim.

(a)



(b)

(c)



(d)

Photograph. 3. (a) and (b).Isolation of bacteria on MRS agar.

Photograph. 3. (c) and (d).Purification of each colony.

6.2. Grouping of representative strains

A total of 120 bacterial isolates were isolated from oil spill (94) and yak milk and its product; churpi (26). MRS medium was used for the purification of the isolates; this was followed by examination of cell morphology and some initial taxonomic tests. Mostly bacteria grew on MRS agar supplemented with 1% $CaCO_3$ under anaerobic condition and formed a clear halo around the colony. The bacteria were catalase negative, gram positive, did not produce carbon dioxide during glucose fermentation and four isolates produced ammonia from arginine.

A grouping of all isolates was based on cell morphology gas production from glucose and ammonia production from arginine (Table 3 and table 4). The representative strains were then selected randomly from each grouped strains having similar morphology, ability to produce gas from glucose and hydrolyse arginine, from the relevant sample sources. The representative strains were assigned their respective strain code number that indicate the name of the samples and source such as OL for oil spill, Y for Yak milk and CH for Yak churpi.

Table 3: Grouping of representative strains of bacteria isolated from Oil spill in North Bengal and Yak milk and its product in Sikkim.

Product	Cell Shape	Glucose Gas from	Arginine hydrolysis	Grouped strains	Representative strains	
					Total code	Strain code
Oil spill	Cocci	-	-	3	76	OL:1, OL:2, OL:3, OL:4, OL:S2, OL:S7, OL:S8, OL:S9, OL:S10, OL:A1, OL:A2, OL:A6, OL:A7, OL:A14, OL:A17, OL:A15, OL:A12, OL:A11, OL:B1, OL:B2, OL:B4, OL:B6, OL:B7, OLB8, OL:B14B, OL:16S, OL:19S, OL:20S, OL:B21M, OL:B22, OL:B23, OL:B24, OL:B25, OL:B26, OL:B15B, OL:B21, OL:B27, OL:C1, OL:C2, OL:C3, OL:C4, OL:C5, OL:C7 OL:C8, OL:C9, OL:C10, OL:C12, OL:C13, OL:C14, OL:C16, OL:C17, OL:C18, OL:C19, OL:C20, OL:D1, OL:D2, OL:D3, OL:D5, OL:D6, OL:D7, OL:D8, OL:D9, OL:D10, OL:D12, OL:D13, OL:D14,OL:D15, OL:D16, OL:D17, OL:D19, OL:D20, OL:D21, OL:D22, OL:D23, OL:D24, OL:D25, OL:D26, OL:D27

	Rod	-	-	1	14	KD:L7, KD:L13 ,KD:L18, OL:A4, OL:A13, OL:B5, OL:B9, OL:B12, OL:B17B, OL:B18, OL:B17S, OL:B20B, OL:C6, OL: C15, OL:D4
	Rod	+	-	0	3	OL:B10, OL:B11, OL:B13
	Cocci	+	-	0	1	D18
Yak milk and yak chur pi	Rod	-	-	3	9	Y:CH3, Y:CH6, Y:CH8, Y:Y2, Y:Y5, Y:Y7, Y:Y9, Y:Y10, Y:CH26, Y:Y1, Y:YB, OL:CH26
	Rod	+	-	3	3	Y: CH7, Y: Yd, Y: CH9.
	Cocci	-	-	3	14	Y:Y6, Y:Y8, Y:Yg, Y:Yh, Y:Yc, Y:Ya, Y:Y4, Y:Y3, Y:CH11, Y:CH5, Y:CH2, Y:CH4, Y:CH10, Y:CH1

6.3. Characteristics and identity of bacteria

Total of 120 bacterial strains isolated from two different sources; oil spill sample of North Bengal and Yak milk and its product churpi from Sikkim, 94 isolates were from oil spill and 26 isolates were from Yak milk and its product churpi. Out of 94 isolates 17 rods from oil spill grew at 45°C along with 7 rods from Yak milk and its product. A total 68 strains were able to grow at 45 ° C and 9 strains from Yak milk Sikkim grew at 45 ° C.

Among all other phenotypic characteristics ability to grow at two different pH of 4.4 and 9.6 were taken into consideration, 15 strains from Oil spill sample and 3 strains from Yak Milk and its isolate had the ability to grow at two different pH. 4 coccoid strains and 2 rod isolates from the oil spill isolates were able to grow at 10 ° C. One of the strain from the Oil spill and two strains from Yak samples were found to be able to grow at both 10°C and 15°C. Ammonia from arginine was produced by four strains from oil spill and three from Yak milk and its product.

Tolerance to salt concentration at 6.5% NaCl was shown by 33 coccoid and 32 rods out of 65 strains from oil spill. Three oil spill isolates showed the ability to grow at both 6.5% and 10% NaCl concentration. The highest salt tolerance of 18% NaCl was shown by the cocci from the oil spill.

Refer to Table 4

Table 4: Phenotypic characteristics of the unidentified strains from vegetable oil spill of North Bengal.

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10° C	15° C	45° C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
KD:L7	small rod	tilted yellow medium	–	–	–	–	–	–	+	+	–	+	–	–
KD:L13	small rod	tilted yellow large	–	–	–	–	–	–	+	+	–	+	–	–
KD:L18	small rod	convex cream medium large	–	–	–	–	–	–	+	+	–	+	–	–
OL:1	small cocci	tilted cream	–	–	–	–	–	–	+	+	–	+	–	–
OL:2	small cocci	large above agar	–	–	–	–	–	–	+	+	–	+	–	–
OL:3	small cocci	tilted cream	–	–	–	–	–	–	+	+	–	–	–	–
OL:4	small cocci	convex cream medium large	–	–	–	–	–	–	-	–	–	+	–	–
OL:S2	small cocci	large above agar	–	–	–	–	–	–	+	+	–	–	–	–
OL:S7	small cocci	large above agar	–	–	–	–	–	–	+	+	–	+	–	–
OL:S8	small cocci	convex tilted	–	–	–	–	–	–	+	+	–	–	–	–
OL:S9	small cocci	convex tilted	–	–	–	–	–	–	+	+	–	–	–	–
OL: A1	small cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	–	–	–
OL: A2	small cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	–	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:A6	cocci	large white convex	–	–	–	–	–	–	+	–	–	+	–	–
OL:A7	cocci	large white convex	–	–	–	–	–	–	+	–	–	+	–	–
OL:A14	cocci	large white convex	–	–	–	–	–	–	+	–	–	–	–	–
OL:A17	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	+	–
OL:A15	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	–	–	–
OL:A12	cocci	large white convex	–	–	–	–	–	–	+	–	–	+	–	–
OL:A11	cocci	large white convex	–	–	–	–	–	–	+	–	–	+	–	–
OL:A13	rod	large white convex	–	–	–	–	–	–	+	–	–	–	–	–
OL:B1	cocci	large white convex	–	–	–	–	–	–	-	+	+	–	–	–
OL:B2	Cocci	large white convex	–	–	–	–	–	–	-	+	–	–	–	–
OL:B3	cocci	large white convex	–	–	–	–	–	–	+	+	+	–	–	–
OL:B4	cocci	tilted white medium	–	–	–	–	–	–	+	+	+	+	–	–
OL:B5	small rod	tilted white medium	–	–	–	–	–	–	–	–	–	–	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10° C	15° C	45° C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:B6	cocci	tilted cream medium	–	–	–	–	–	–	+	–	+	+	–	–
OL:B7	cocci	tilted cream medium	–	–	–	–	–	–	+	+	–	+	–	–
OL:B8	cocci	large white convex	–	–	–	–	–	–	+	+	–	–	–	–
OL:B9	rod	large white convex	–	–	–	–	–	–	+	+	–	–	–	–
OL:B10	slender rods	tilted cream medium	+	–	–	–	–	–	+	+	–	+	+	–
OL:B11	rod	tilted cream medium	+	–	–	–	–	–	+	+	–	–	–	–
OL:B12	thread like rod	tilted cream medium	–	–	–	–	–	–	+	+	–	+	–	–
OL:B13	cocci	large white convex	+	–	–	–	–	–	+	–	–	–	–	–
OL:B14B	cocci	tilted cream medium	–	–	–	–	–	–	+	+	+	–	–	–
OL:B16S	thread like rod	tilted cream medium	–	–	–	–	–	–	+	+	+	+	+	–
OL:B17B	rod	tilted cream medium	–	–	–	–	–	–	+	–	–	+	+	–
OL:B18	rod	tilted cream medium	–	–	–	–	–	–	+	–	–	–	–	–
OL:19S	cocci	tilted white medium	–	–	–	–	–	–	-	+	+	+	+	–
OL:20S	cocci	tilted white medium	–	–	–	–	–	–	–	–	–	+	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:21M	cocci	large white convex	–	–	–	–	–	–	–	–	–	+	–	–
OL:B22	cocci	tilted cream medium	–	–	–	–	–	–	–	–	+	+	+	–
OL:B23	cocci	tilted cream medium	–	–	–	–	+	–	–	+	+	+	+	–
OL:B24	cocci	tilted cream medium	–	–	–	–	+	–	-	+	+	+	–	–
OL:B25B	cocci	large white convex	–	–	–	–	–	–	+	–	–	–	+	+
OL:B25S	cocci	large white convex	–	–	–	–	–	–	+	+	+	–	–	–
OL:B26	cocci	large white convex	–	–	–	–	–	–	+	+	+	–	–	–
OL:B15B	cocci	large white convex	–	–	–	–	+	–	+	+	+	+	–	–
OL:B17S	rod	large white convex	–	–	–	–	–	–	+	–	–	+	–	–
OL:B20B	Rod	large white convex	–	–	–	–	–	–	+	–	+	+	–	–
OL:B21	Cocci	large cream convex	–	–	–	–	–	–	+	–	–	+	+	–
OL:B27	Cocci	large cream convex	–	–	–	–	–	+	+	+	–	+	–	–
OL:C1	Cocci	tilted medium white convex	–	–	–	–	–	–	+	+	+	+	–	–
OL:C2	Cocci	tilted medium white convex	–	–	–	–	–	–	–	–	–	+	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:C3	cocci	tilted medium white convex	–	–	–	–	–	–	+	+	–	+	–	–
OL:C4	cocci	tilted medium white convex	–	–	–	–	–	–	+	+	–	+	–	–
OL:C6	rod	large white convex	–	–	–	–	+	–	+	+	–	+	–	–
OL:C7	cocci	large white convex	–	–	–	–	–	–	+	+	–	–	–	–
OL:C8	cocci	large white convex	–	–	–	–	–	–	+	+	–	+	–	–
OL:C9	cocci	tilted cream medium	–	–	–	–	–	–	+	+	–	–	–	–
OL:C10	cocci	tilted cream medium	–	–	–	–	–	–	+	–	+	–	–	–
OL:C11	cocci	tilted cream medium	–	–	–	–	–	–	–	+	–	–	–	–
OL:C12	cocci	tilted cream medium	–	–	–	–	–	–	–	+	+	–	–	–
OL:C13	cocci	tilted cream medium	–	–	–	–	–	–	–	+	–	–	–	–
OL:C15	rod	large white convex	–	–	–	–	–	–	–	+	–	–	–	–
OL:C16	cocci	large white convex	–	–	–	–	–	–	–	+	+	–	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:C17	cocci	tilted cream medium	–	–	–	–	–	–	–	+	–	–	–	–
OL:C18	cocci	tilted cream medium	–	–	–	–	–	–	–	+	–	+	–	–
OL:C19	cocci	tilted cream medium	–	–	–	–	–	–	–	+	+	+	–	–
OL:C20	cocci	tilted cream medium	–	–	–	–	–	–	–	+	–	+	–	–
OL:D1	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–
OL:D2	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–
OL:D3	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D4	rod	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D5	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D6	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D7	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D8	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D9	cocci	tilted cream medium	–	–	–	–	–	–	+	–	+	+	–	–
OL:D10	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:D11	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D12	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D13	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D14	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–
OL:D15	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	+	–
OL:D16	cocci	tilted cream medium	–	–	–	–	–	–	–	–	+	+	+	–
OL:D17	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D18	cocci cocci	tilted cream medium	+	–	–	–	–	–	+	–	–	+	+	–
OL:D20	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D21	cocci	tilted cream medium	–	–	–	–	–	–	+	–	–	+	–	–
OL:D22	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–
OL:D23	cocci	tilted cream medium	–	–	–	–	–	–	+	–	+	+	–	–
OL:D24	cocci	tilted cream medium	–	–	–	–	–	–	+	–	+	+	–	–

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
OL:D25	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–
OL:D26	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–
OL:D27	cocci	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–

Table: Table: Phenotypic characteristics of the unidentified strains from Yak milk and Yak milk product (churpi) of North Sikkim.

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in							
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%
CH:2	cocci	tilted medium white convex	–	–	–	–	–	–	+	–	–	–	–	–
CH:3	rod	large white convex	–	–	–	–	–	–	+	–	+	–	–	–
CH:5	cocci	large white convex	–	–	–	–	–	–	+	+	+	–	–	–
CH:6	rod	tilted cream medium	–	–	–	–	–	–	+	–	+	–	–	–
CH:8	rod	tilted cream medium	–	–	–	–	–	–	–	–	+	–	–	+
CH:11	cocci	tilted cream medium	–	–	–	–	–	–	–	–	+	–	–	–
Y:Y2	rod	tilted cream medium	–	–	–	–	–	–	–	–	+	–	–	–
Y:Y3	cocci	large white convex	–	–	–	–	–	–	–	–	+	–	–	–
Y:Y4	cocci	large white convex	–	–	–	–	–	–	–	–	+	–	–	–
Y:Y5	rod	large white convex	–	–	–	–	–	–	–	–	+	–	–	–
Y:Ya	cocci	large white convex	–	–	–	–	–	–	–	–	+	–	–	–

Y:Yc	cocci	large white convex	–	–	–	–	–	–	–	–	+	–	–	–	
Y:Yh	cocci	large white convex	–	–	–	–	–	–	–	+	+	–	–	–	
Y:Yg	cocci	tilted cream medium	–	–	–	–	–	–	–	–	+	–	–	–	

Y:Y6	cocci	tilted medium white convex	–	–	–	–	–	–	–	+	–	–	–	–	
Y:Y7	rod	large white convex	–	–	–	–	–	–	–	+	–	+	–	–	
Y:Y8	cocci	large white convex	–	–	–	–	–	–	–	+	+	+	–	–	
Y:Y9	rod	tilted cream medium	–	–	–	–	–	–	–	+	–	+	–	–	
Y:Y10	rod	tilted cream medium	–	–	–	–	–	–	–	–	–	+	–	–	
Y:Y11	rod	tilted cream medium	–	–	–	–	–	–	–	+	–	+	–	–	

Table : Phenotypic characteristics of the identified strains from vegetable oil spill of North Bengal and Yak milk and its product (churpi) of North Sikkim.

Strain code	Cell Morphology	Colony Morphology	NH_3 from arginine	CO_2 from glucose	Catatalase test	KOH Test	Growth at/in								Sugars Fermented							Identity
							10°C	15°C	45°C	pH 4.4	pH 9.6	NaCl 6.5%	NaCl 10%	NaCl 18%	Xylose	Galactose	Sucrose	Xylose	Ttrehalose	Arabinose	Raffinose	
OL:C5	cocci	Medium white tilted	–	–	–	–	–	–	+	+	+	+	–	–	+	+	+	+	+	+	+	*Dermabacter hominis*
OL:C14	cocci	Large white convex	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+	+	+	+	+	*Enterococcus hirae*
OL:A4	rod	Medium White	–	–	–	–	+	+	–	–	+	–	–	–	+	+	+	+	+	+	+	*Paenibacillus cineris*
OL:D19	cocci	Large white convex	–	–	–	–	–	–	+	–	–	–	–	–	+	+	+	+	+	+	+	*Bacillus cereus*
Y:CH4	cocci	Medium white tilted	–	–	–	–	+	–	–	+	–	+	–	–	–	+	–	+	+	+	+	*Enterococcus durans*

Y:CH9	rod	Medium white tilted	+	–	–	–	–	+	+	+	–	+	–	–	–	+	–	+	+	+	+		*Lactobacillus fructivorans*
Y:CH7	rod	Medium White	+	–	–	–	–	+	+	+	–	+	–	–	+	+	+	+	+	+	+		*Lactobacillus fructivorans*
Y:CH10	cocci	Large white convex	–	–	–	–	+	–	–	–	+	+	–	–	+	+	+	+	+	+	+		*Enterococcus casseliflavus*
Y:CH1	cocci	Large white convex	–	–	–	–	+	–	–	–	+	+	–	–	+	+	+	+	+	+	+		*Enterococcus casseliflavus*
Y:CH26	rod	Medium White	–	–	–	–	+	+	–	+	–	–	–	–	+	+	+	+	+	+	+		*Paenibacillus tundrae*
Y:Yd	rod	Medium White	+	–	–	–	–	+	+	+	–	+	–	–	+	+	+	+	+	+	+		*Lactobacillus fructivorans*
Y:Yb	rod	Large white convex	–	–	–	–	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+		*Gracibacillus halotolerans*

Y:Y1	rod	Large white convex	–	–	–	–	–	+	+	–	+	–	–	–	+	+	+	+	+	+	+	*Lysinibacillus boronitolerans*

6.4. Screening for Cholesterol utilization

The bacterial isolates were screened for cholesterol utilization followed by checking quantification of the respective strains using cholesterol kit. The control tube contained medium A supplemented with cholesterol which was free of inoculums. After incubation of the broth in triplicates with the respective strains, quantification assay was done and optical density was taken in UV-VIS spectrophotometer. The cholesterol percentage was calculated using the formula:

Cholesterol Concentration (mg/dl) = Absorbance of Test / Absorbance of standard*100

Table 4 and Table 5 show the percentage of cholesterol of the bacterial strains isolated from oil spill and Yak milk and its product. Out of 120 isolates 13 (OL:C5,OL:C14,OL:A4,OL:D19,Y:CH4,Y:CH9,Y:CH7,Y:CH10,Y:CH1,Y:CH26, Y:Yd,Y:Yb,Y:Y1)
showed cholesterol lowering property and only those strains were identified, four isolates (OL:C5,OL:C14,OL:A4,OL:D19) from oil spill showed 10.34± 0.180, 10.88±0.016, 11.46±0.377, 11.96±0.138 cholesterol concentration. *Dermabacter hominis* OL: C5 showed lower percentage concentration of 10.34± 0.180 as compared to the control tube having 13.33±0.030 %. Similarly, 26 isolates from Yak milk and its product had in between 10.16% - 11. 98%. The lowest percentage was shown by *Lactobacillus fructivorans* Y: CH7. Three strains 2 from Yak churpi (Y: CH9, Y: CH7) and 1(Y: Yd) from yak milk was identified as *Lactobacillus fructivorans*, others were *Lysinibacillus boronitolerans* Y: Y1(milk) and *Gracibacillus halotolerans* Y: Yb (milk).

Paenibacillus tundra Y: CH26, 2 strains of *Enterococcus casseliflavus* Y: CH10; Y: CH1 and *Enterococcus durans* Y: CH4 were identified from Yak churpi. In the same way, 4 strains *Dermabacter hominis* OL: C5, *Enterococcus hirae* OL: C14, *Paenibacillus cineris* OL: A4, *Bacillus cereus* OL: D19 were identified from oil spill sample.

Table 4: The Cholesterol Estimation of 20 isolates from Oil Spill of North Bengal.

Strain code	Average Percentage of Cholesterol Concentration
CONTROL	13.33±0.030
OL:C18	11.99±0.020
OL:S7	12.56±0.010
OL:D22	12.06±0.001
OL:D24	11.98±0.030
OL:C4	11.99±0.041
OL:3	12.35±0.066
OL:1	12.02±0.10
OL:C16	11.99±0.001
OL:B8	12.89±0.009
OL:B15B	12.76±0.005
OL:A14	12.59±0.160
OL:B9	12.70±0.450
OL:S8	12.90±0.091
OL:D26	12.83±0.083
OL:D27	12.78±0.050
OL:D1	12.01±0.066
OL:C5	10.34± 0.180
OL:C14	10.88±0.016
OL:A4	11.46±0.377
OL:D19	11.96±0.138

Table 5: Cholesterol Estimation of 20 isolates from yak milk and yak milk product (churpi).

Strain code	Percentage of Cholesterol Concentration (mg/dl)
CONTROL	13.33±0.030
CH:2	12.99±0.020
CH:3	12.56±0.010
CH:5	12.06±0.001
CH:6	12.98±0.030
CH:8	11.99±0.041
CH:11	11.35±0.066
Y: CH4	11.27±0.017
Y: CH1	11.77±0.142
Y: CH9	11.94±0.052
Y: Yd	11.94±0.23
Y:Y9	12.59±0.160
Y:Yg	12.70±0.450
Y: CH26	11.05±0.098
Y:Y7	12.83±0.083
Y: Yb	11.198±0.27
Y:Y11	12.01±0.066
Y: CH7	10.16±0.017
Y:Yg	12.88±0.016
Y: Y1	11.04±0.015
Y: CH10	11.98±0.024

Table 6 : Percentage of Cholesterol utilization of identified strain from oil spill, Yak milk and Yak churpi isolates .

Product	Code	Strain	% of Cholesterol Concentration
Oil Spill	Control		13.33±0.030
	OL: C5	*Dermabacter hominis*	10.34± 0.180
	OL: C14	*Enterococcus hirae*	10.88±0.016
	OL: A4	*Paenibacillus cineris*	11.46±0.377
	OL: D19	*Bacillus cereus*	11.96±0.138
Yak Churpi	Y: CH4	*Enterococcus durans*	11.27±0.017
	Y: CH9	*Lactobacillus fructivorans*	11.94±0.052
	Y: CH7	*Lactobacillus fructivorans*	10.16±0.017
	Y: CH10	*Enterococcus casseliflavus*	11.98±0.024
	Y: CH1	*Enterococcus casseliflavus*	11.77±0.142
	Y: CH26	*Paenibacillus tundra*	11.05±0.098
Yak milk	Y: Yd	*Lactobacillus fructivorans*	11.94±0.23
	Y: Yb	*Gracibacillus halotolerans*	11.198±0.27
	Y: Y1	*Lysinibacillus boronitolerans*	11.04±0.015

6.5.1. Acid tolerance of bacterial strains

Table 7 shows the percentage of acid tolerance of the identified bacterial strains. Out of 13 strains identified all of them showed 98% acid tolerance activity in pH 3. Among them one strain, *Lactobacillus fructivorans* showed 99% acid tolerance.

Table 7: Percentage of Acid tolerance of identified strain from oil spill, Yak milk and Yak churpi isolates.

Product	Code	Strain	% of Acid Tolerance Concentration
Oil Spill	OL: C5	*Dermabacter hominis*	98.89±0.01
	OL: C14	*Enterococcus hirae*	98.96±0.05
	OL: A4	*Paenibacillus cineris*	99.30±0.00
	OL: D19	*Bacillus cereus*	97.90±0.01
Yak Churpi	Y: CH4	*Enterococcus durans*	98.76±0.03
	Y: CH9	*Lactobacillus fructivorans*	99.82 ±0.01
	Y: CH7	*Lactobacillus fructivorans*	98.77 ±0.01
	Y: CH10	*Enterococcus casseliflavus*	98.75±0.01
	Y: CH1	*Enterococcus casseliflavus*	99.15±0.01
	Y: CH26	*Paenibacillus tundra*	98.98±0.01
Yak milk	Y: Yd	*Lactobacillus fructivorans*	99.89±0.00
	Y: Yb	*Gracibacillus halotolerans*	98.87±0.01
	Y: Y1	*Lysinibacillus boronitolerans*	98.89±0.01

6.5.2. Bile tolerance of the identified strains

After exposure to acidic conditions, 13 selected acidotolerant isolates were assayed for bile salt tolerance. The isolates showed bile tolerance as growth was observed at 0.4% bile salt concentration. However three isolates *Dermabacter homins* OL: C5, *Enterococcus durans* Y: CH4 and *Gracibacillus halotolerans* Y: Yb gave 47.08%, 30.79%, 36.59% of Bile tolerating activity.

Table 8: Percentage of Bile tolerance of identified strain from oil spill, Yak milk and Yak churpi isolates.

Product	Code	Strain	% of Bile Tolerance Concentration
Oil Spill	OL: C5	*Dermabacter hominis*	47.08±8.63
	OL: C14	*Enterococcus hirae*	17.79±0.28
	OL: A4	*Paenibacillus cineris*	20.40±0.70
	OL: D19	*Bacillus cereus*	15.08±0.50
Yak Churpi	Y: CH4	*Enterococcus durans*	41.15± 0.18
	Y: CH9	*Lactobacillus fructivorans*	30.79±0.51
	Y: CH7	*Lactobacillus fructivorans*	18.87±6.61
	Y: CH10	*Enterococcus casseliflavus*	17.33±0.12
	Y: CH1	*Enterococcus casseliflavus*	13.05±0.39
	Y: CH26	*Paenibacillus tundra*	21.68±0.09
Yak milk	Y: Yd	*Lactobacillus fructivorans*	8.01±0.75
	Y: Yb	*Gracibacillus halotolerans*	36.59±3.40
	Y: Y1	*Lysinibacillus boronitolerans*	14.14±0.63

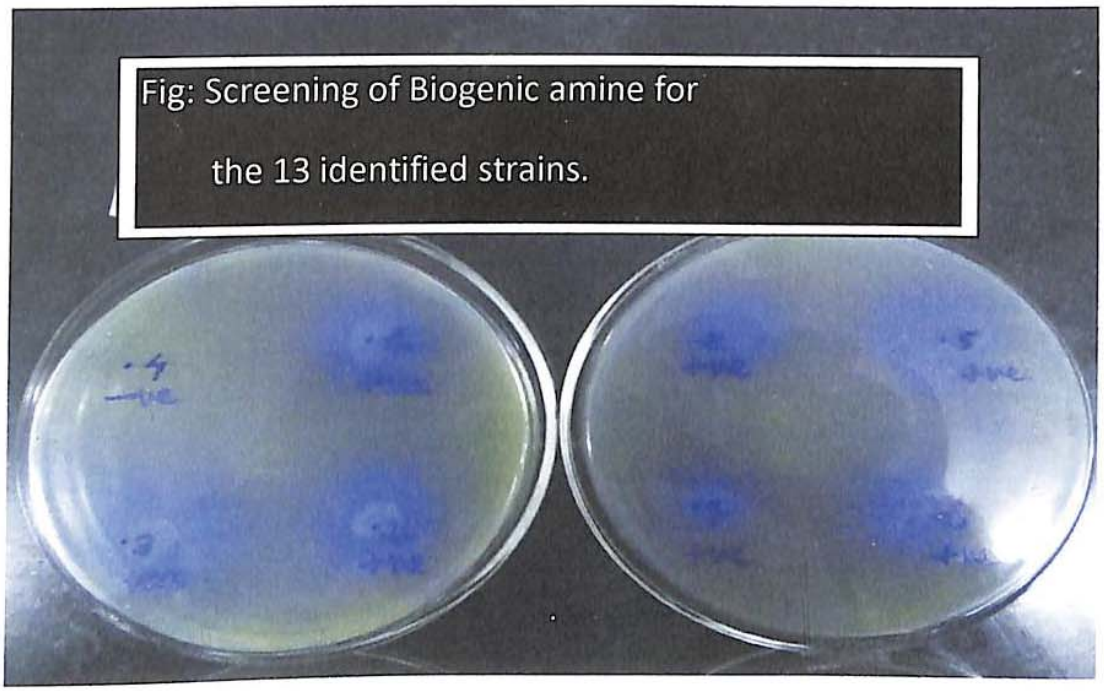
6.5.3.Screening of Biogenic Amines producing bacteria

The strains of bacteria isolated from oil spill, Yak milk and Yak churpi were screened for their ability to produce biogenic amines (Table 9).Most of the strains did not produce biogenic amines with the method applied. Results were positive in plates containing Tyrosine however one strain *Enterococcus casseliflavus and Enterococcus durans* showed negative result. The *Lactobacillus* strain gave negative result for amino acid decarboxylase activity. None of the strains of *Enterococcus ,Paenibacillus, Lysinibacillus* and *Gracibacillus* gave positive result for the production of biogenic amine in this method. No biogenic amine was detected in plates with Lysine and Histidine so the strains showed no decarboxylase activity.

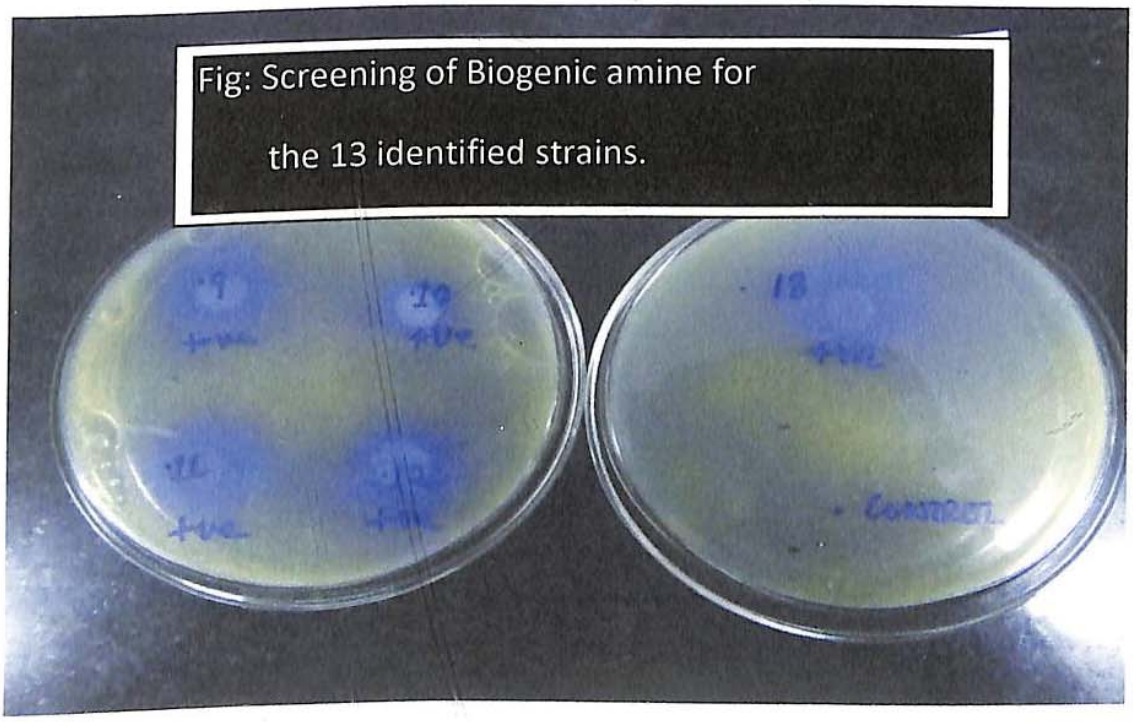
Table 9: Screening of biogenic amine producing strains from Yak milk and Yak churpi isolates.

Product	Code	Strain	Amino acids		
			Tyrosine	Lysine	Histidine
Oil Spill	OL: C5	*Dermabacter hominis*	+	-	-
	OL: C14	*Enterococcus hirae*	+	-	-
	OL: A4	*Paenibacillus cineris*	+	-	-
	OL: D19	*Bacillus cereus*	+	-	-
Yak Churpi	Y: CH4	*Enterococcus durans*	-	-	-
	Y: CH9	*Lactobacillus fructivorans*	+	-	-
	Y: CH7	*Lactobacillus fructivorans*	+	-	-
	Y: CH10	*Enterococcus casseliflavus*	-	-	-
	Y: CH1	*Enterococcus casseliflavus*	-	-	-
	Y: CH26	*Paenibacillus tundra*	+	-	-
Yak milk	Y: Yd	*Lactobacillus fructivorans*	+	-	-
	Y: Yb	*Gracibacillus halotolerans*	+	-	-
	Y: Y1	*Lysinibacillus boronitolerans*	+	-	-

Tyr = Tyrosine, tyramine precussor; Lys = Lysine, cadaverine precussor; His =Histidine , histamine precussor .



(a)



(b)

Photograph 4(a).and (b).Screening of Biogenic amine Production.

No.1 to 13 in the plates denote the identified strains in a chronological order as in table 9.

6.5.4. Hydrophobicity of the bacterial strains

Table 10 show the percentage of hydrophobicity of the bacterial strains. All 13 strains show less hydrophobicity. Among 13 isolates *Paenibacillus cineris* OL: A4 shows the highest percentage of 4.481%. Other 12 strains did not show as much hydrophobicity as that of *Paenibacillus cineris* OL: A4.The range of percentage of hydrophobicity was 0.23% to 1.87%.

Table 10: Percentage of Hydrophobicity of identified strain from oil spill, Yak milk and Yak churpi isolates.

Product	Code	Strain	% of Hydrophobicity
Oil Spill	OL: C5	*Dermabacter hominis*	1.87±0.24
	OL: C14	*Enterococcus hirae*	1.20±0.16
	OL: A4	*Paenibacillus cineris*	4.48±0.26
	OL: D19	*Bacillus cereus*	0.03±0.03
Yak Churpi	Y: CH4	*Enterococcus durans*	1.29±0.17
	Y: CH9	*Lactobacillus fructivorans*	0.91±0.15
	Y: CH7	*Lactobacillus fructivorans*	0.23±0.15
	Y: CH10	*Enterococcus casseliflavus*	0.37±0.09
	Y: CH1	*Enterococcus casseliflavus*	0.09±0.03
	Y: CH26	*Paenibacillus tundra*	0.28±0.05
Yak milk	Y: Yd	*Lactobacillus fructivorans*	0.59±0.08
	Y: Yb	*Gracibacillus halotolerans*	0.79±0.06
	Y: Y1	*Lysinibacillus boronitolerans*	0.15±0.13

6.5.5. Antimicrobial activities

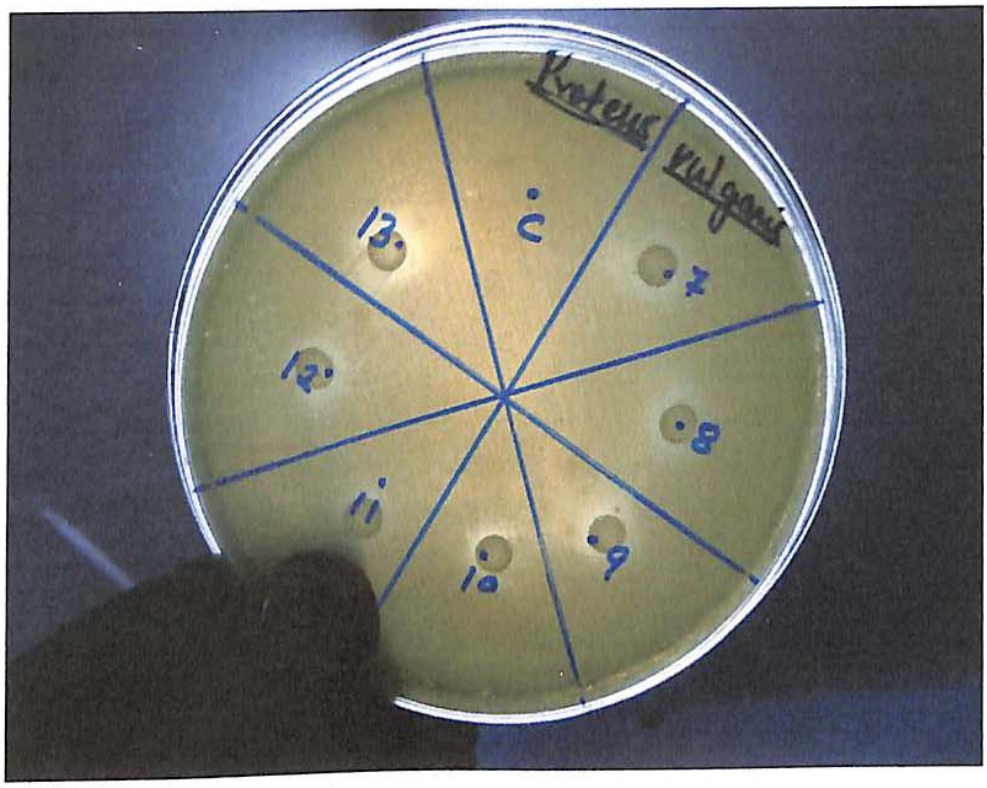
Table 11 shows the antagonistic activity of the 13 bacterial strains, isolated from oil spill, Yak milk and Yak churpi isolates against different indicator strains, *Lactobacillus plantrum* Ek4, *Lactobacillus acidophilus, Bacillus subtilis, E.coli, Pediococcus pentosus* R1B1, *Lactobacillus casei* Ek 35. Most of the identified strains showed the clear inhibition zones in agar spot test against the indicator strains, showing antagonism. The results were negative in case of the indicator strains where , *Lactobacillus plantrum* Ek4, *Lactobacillus acidophilus, Bacillus subtilis, E.coli, Pediococcus pentosus* R1B1, *Lactobacillus casei* Ek 35 were used. However in plate where *Proteus vulgaris* 742 was used as an indicator the results were positive with 12 strains namely; *Dermabacter hominis* OL: C5, *Enterococcus hirae* OL: C14, *Paenibacillus cineris* OL: A4 , *Bacillus cereus* OL: D19 , *Enterococcus durans* Y: CH4, *Lactobacillus fructivorans* Y:CH9, Y:Yd, Y: CH7(3),*Paenibacillus tundra* Y: CH26, *Gracibacillus halotolerans* Y: Yb and *Lysinibacillus boronitolerans* Y: Y1. Two strains *Enterococcus casseliflavus* Y: CH1, Y: CH10 (2) and *Paenibacillus tundra* Y: CH26 showed negative result having no inhibition zone. About 4mm inhibition zone was seen in case of four isolates (CH7, CH9, CH, Yd), 2mm to 4 mm inhibition zone was seen in case of 6 strains (C5, C14, A4, D19, Yb).

Table 11: Antimicrobial activity of the identified strains from oil spill, Yak milk and Yak churpi isolates.

Strains	Indicator Strains						
	Proteus vulgaris	*Lactobacillus acidophilus*	*Lactobacillus plantrum*	*Bacillus subtilis*	*E.coli*	*Pediococcus pentosus*	*Lactobacillus casei*
Oil Spill and Yak samples							
OL: C5	+	-	-	-	-	-	-
OL: C14	+	-	-	-	-	-	-
OL: A4	+	-	-	-	-	-	-
OL: D19	-	-	-	-	-	-	-
Y: CH4	++	-	-	-	-	-	-
Y: CH9	++	-	-	-	-	-	-
Y: CH7	++	-	-	-	-	-	-
Y: CH10	+	-	-	-	-	-	-
Y: CH1	+	-	-	-	-	-	-
Y: CH26	-	-	-	-	-	-	-
Y: Yd	++	-	-	-	-	-	-
Y: Yb	+	-	-	-	-	-	-
Y: Y1	+	-	-	-	-	-	-

Data shows sets of experiments.

-, no zone of inhibition ; +,2mm □zone □ 4mm;++ 4mm□zone □6mm+++,□6mm



Photograph 5.Test for antimicrobial activity.

n the plate, No. 13, 9 and 10 denotes Lb. *L .Fructivorans CH9, Lb .Fructivorans* CH7, *Dermabacter hominis* C5 respectively

DISCUSSION

Microorganisms

The microbial population of Yak Milk, its product and vegetable oil spill samples revealed that lactic bacteria comprised of Lactobacilli and *Enterococcus* along with other genera like *Paenibacillus*, *Lysinibacillus*, *Gracibacillus* and *Dermabacter*. Among the identified ones, and other bacteria were the predominant microorganisms present in viable numbers above10^8 cfu/g. The biochemical characterization of the 120 isolates was carried out based on morphology, gas production from glucose fermentation, and growth at different temperatures (Mundt, 1986). In all the Oil Spill samples the population of LAB as well as other aerobes were in the range of 10^6 to 10^8 cfu /ml. The count of LAB in Yak milk sample was less being 10^5 cfu/ ml. All bacterial isolates were tentatively identified as LAB because they were Gram-positive, catalase-negative bacteria which did not form spores and were non-motile and form halo around the colony in the MRS Agar supplemented with 1% $CaCO_3$. The strains of LAB were grouped on the basis of cell morphology, production of gas from glucose and hydrolysis of arginine and the representative strains were randomly selected from each group. Representative strains were phenotypically characterized by observing growth at different temperatures (10, 15 and 45 ° C), pH (3.9 and 9.6) and salt tolerance (NaCl 6.5, 10 and 18%). Earlier reports showed the domination of lactobacilli in chhurpi and chhu of Sikkim (Tamang *et al.* 2000; Dewan and Tamang 2007). It was observed that rods only were detected in philu, made from yak milk suggesting that the milk type is the main source for the flora present in philu and so yaks milk products may have a distinctive flora. Species of LAB recovered in the Himalayan milk products corresponds with that of LAB typically reported for dairy products of other geographical regions (Abdelgadir *et al.* 2001; Beukes *ét al.* 2001; Xanthopoulos 2001; Gran *et al.* 2003; Mathara *et al.* 2004;Chammas *et al.* 2006; Zamir *et al.* 2006).

Probiotic properties

Weight gain and obesity are the main physiological problem in the world. Increase in weight causes many disorders, among which peripheral artery disease, coronary disease, hypertension and cardiovascular diseases are the main. Cardiovascular diseases (CVD) are the main cause of disability. The diseases associated with

cholesterol are the topmost cause of morbidity and mortality in industrialized countries in the twentieth century. The main component of CVD is cholesterol. There have been reports where consumption of fermented milk has reduced cholesterol level in humans (Mann *et al.,* 1974). There are reports that Lactic acid bacteria (LAB) especially *Lactobacillus acidophilus* have cholesterol reducing property (Gilliland *et al.,* 1985; Danielson *et al.*, 1989; Lin *et al.*, 1989; Fukushima and Nakano 1996). In the present study thirteen strains have shown significant cholesterol lowering property from the laboratory media during growth conditions. Among them three strains *Lactobacillus fructivorans* Y: CH7, *Dermabacter hominis* OL: C5 and *Enterococcus hirae* OL: C14 were identified that showed more cholesterol lowering activity than others ranging from 10.16 to 10.88% as compared to 13.33 % in the control tube. In order to administer these strains it is necessary to check their acid and bile tolerance activity and the mechanism of cholesterol removal.

Study of technological properties of LAB strains isolated from fermented milk products is an important criterion for selection of starter cultures to be used in the manufacture of dairy products (Durlu- Ozkaya *et al.* 2001; Badis *et al.* 2004). The role of probiotic organisms as alternative or complementary therapy in combating large number of gastro intestinal disorders and their ability to enhance immune response attracts global attention. In addition, their therapeutic use towards cholesterol-lowering activities has further increased their applications as effective probiotics for humans as supplements in milk and yoghurt, since there are no other supplements for hypercholesterolemia, which is the crucial risk factor for cardiovascular diseases (Sudha *et al*., 2009).

Probiotics are microorganisms which provide health promoting effects. The criterion used to select potential probiotics is related to cholesterol metabolism, acid and bile tolerance and production of antimicrobial substances for food and clinical use (Ouwehand *et al.,* 1999). The survival of the bacterial strains under experimental conditions is to indicate their survival passage through their stomach with pH between 1.5 and 3.0 (Corzo and Gilliland, 1999). The 13 identified strains did not show acid tolerance at pH 2 but the strains displayed growth at a pH of 3.The acid tolerance ranged from 97-99% among the identified bacterial strains. Studies have reported that tolerance to acid and other gastrointestinal stresses is specific to the strain (Morelli, 2000; Huang and Adams, 2004). In order to survive and colonize the

gastrointestinal tract, microorganisms should express tolerance to acid and bile salts (Gibson, 1998). The characteristics of a food including the pH in which potential probiotics are delivered into the gut may have a buffering effect and significantly influence survival of the microorganisms (Patel et al., 2004).The results suggests that all strains would transit the pH of the human stomach and function effectively in that environment.

The bile tolerance is the second criteria for the selection of probiotics. The resistance to bile salts is considered to be an essential property for the strain to survive under the conditions in the small intestine. Bile salts are synthesised from the cholesterol in the liver and are secreted from the gall bladder into the duodenum in the conjugated form in volumes ranging from 500 to 700ml per day (Hoffman *et al.,* 1983). The concentration of human bile range that is relevant under physiological condition is from 0.1 to 0.3% (Dunne *et al.,* 2001) and 0.5% (Mathara *et al.,* 2008). Therefore, it is needed that efficient probiotic bacteria should be able to grow in bile salt with concentration ranging from 0.15 - 0.30% (w/v) (Suskovi et al., 2000).Out of 13 isolates three isolates *Dermabacter homins* OL: C5, *Enterococcus durans* Y: CH4 and *Gracibacillus halotolerans* Y: Yb and *Lactobacillus fructivorans* Y: CH9 gave 47.075%, 30.79%, 41.15%, 36.59% of Bile tolerance activity. These percentages are however not considered as good for tolerance, since if the isolates did not give 50% of activity although they survived at 0.4% bile salt after 24 hour.

Antagonostic properties of the identified bacterial strains were tested against *Lactobacillus plantrum* Ek4, *Lactobacillus acidophilus, Bacillus subtilis, E.coli, Pediococcus pentosus* R1B1, *Lactobacillus casei* Ek 35 and *Proteus vulgaris*. Most of the bacterial strains showed antagonistic property against *Proteus vulgaris.* This reveals that antimicrobial activity of the identified strains can reduce the number of other undesired microorganisms. In addition, LAB competes with other microbes by screening antagonistic compounds and modifying the microenvironment by their metabolism (Lindgren and Dobrogosz, 1990).

Biogenic amines (BA) are low molecular weight compounds occurring naturally that are involved in various biological activities in most living organisms. BA can however also trigger human health problems leading to vomiting, hypertension, headaches and palpitations, during food consumption (Silla, 1996). During

fermentation process the Lactic acid bacteria are capable of converting the amino acid precursors into BA by decarboxylation or deamination. For this reason the biogenic amine producing bacterial strains had to be screened for safety.

The two most important biogenic amine in food studied are with respect to histamine and tyramine due to their toxicological effects. In the present study tyramine, cadaverine and histamine were investigated for the production of biogenic amine. Table 9 shows the production of biogenic amines by the identified strains. Tyramine levels were high compared to the others which may be due to the composition of decarboxylase medium, the incubation time and the strain being assayed. Out of 13, 11 were found to be tyrosine decarboxylase species. No strain produced cadaverine and histamine. The ability to decarboxylate amino acids is generally considered a strain-dependent characteristic rather than a species property. Recent studies suggest new interesting hypothesis on the physiological role of amine in microorganisms. Some strains, which possess amino acid decarboxylase activity, could be able overcome or reduce the effects of temperature, NaCl, and other biological and chemico-physical factors that induce stress responses in the cells, with the production of some BA. (Schiller *et al.*, 2000).

CONCLUSION

Cardiovascular diseases are a major physiological problem worldwide. It is the most important cause of death in westernized countries including India. In India it is one of the top ten causes of death. Hypercholesterolemia is strongly associated with coronary heart disease and arteriosclerosis, and decreasing serum cholesterol is an important treatment option. The source of cholesterol is dietary cholesterol and also the cholesterol synthesised in the liver. The LDL-cholesterol causes accumulation of cholesterol in blood vessels leading to arterosclerosis. According to Frick et al., (1987), every 1% reduction in body cholesterol content lowers the risk for cardiovascular diseases by 2%.Change in lifestyle and dietary habits contribute to the rise in the level of cholesterol. The consumption of food like the fermented food have reported (Mann and Spoerry., 1974), the cholesterol-lowering potential of lactic acid bacteria such as Lactobacillus and Bifidobacterium were studied. Taking the earlier reports as a base two sample sources were chosen with the assumption that cholesterol utilizing bacteria may be present there. However it can be said that Yak Milk and its fermented product and Oil Spill are good sources of cholesterol utilizing lactic acid bacteria. Among the 13 strains identified, three strains *Lactobacillus fructivorans* Y: CH7, *Dermabacter hominis* OL: C5 and *Enterococcus hirae* OL: C14, in particular have shown significant cholesterol lowering property. The Yak milk and the churpi sample from the Northern region of Sikkim contained nine cholesterol reducing bacteria and the Oil spill isolates contain four .The cholesterol lowering bacteria screened were identified belonging to varied considerably in their genus, *Lactobacilli* and *Enterococcus* along with other genera like *Paenibacillus, Lysinibacillus, Gracibacillus* and *Dermabacter.* The identified strains were studied for other probiotic properties like acid tolerance, bile tolerance, hydrophobicity assay, production of biogenic amine and test for antimicrobial activity. Out of 13 strains identified all of them showed 98% acid tolerance activity in pH 3. Among them one strain, *Lactobacillus fructivorans* showed 99% acid tolerance. However four isolates *Dermabacter homins* OL: C5, *Enterococcus durans* Y: CH4 *Gracibacillus halotolerans* Y: Yb and *Lactobacillus fructivorans* Y: CH9 gave 47.075%, 41.15%, 30.79%, 36.59% of Bile tolerating activity. All 13 strains show less hydrophobicity being less than 50%. Results were positive in plates containing Tyrosine, however two strains *Enterococcus durans* and *Enterococcus caselliflavus* showed negative result. No biogenic amine was detected in plates with Lysine and Histidine so the strains showed no decarboxylase activity. Two strains

Enterococcus casseliflavus Y: CH1, Y: CH10 (2) and *Paenibacillus tundra* Y: CH26 showed negative result having no inhibition zone while all other strains 11 strain showed positive antimicrobial activity against *Proteus vulgaris*. After detecting the probiotic property it can be concluded that *Enterococcus durans* (OL:CH4), *Lactobacillus fructivorans* (OL:CH7, CH9,Yd), and *Enterococcus casseliflavus* CH10 and CH1 can be studied with more emphasis on the cholesterol utilizing activity since they show good acid tolerance, bile tolerance, negative and result for biogenic amine production.

SUMMARY

The major objective of this thesis was twofold: (a) the isolation of the cholesterol utilizing bacteria from the yak milk sample and fermented milk product or churpi of yak from Lachung and from Changu, North Sikkim; and oil spill samples from vegetable oil industry or packing industry in Paribhanagar, Matigara and from Jalpaiguri, North Bengal, and (b) the probiotic properties of the cholesterol utilizing bacterial strains to know their safety measures for consumption. During the first phase considerable procedure for isolation of various bacteria was performed on MRS agar after sample collection. Some knowledge on the health benefits of Yak milk, churpi preparation and production and packaging of mustard oil in the industry was obtained at the time of sample collection.

A chief emphasis is this thesis has been on the screening of the cholesterol lowering bacterial strains with regard to cholesterol utilization. For the Oil spill sample the population of LAB as well as other aerobes in the range of 10^6 to 10^8 cfu /ml were detected. The count of bacteria on MRS agar in Yak milk sample was less being 10^5 cfu/ ml. Bacteria encircled with halo which could not be confirmed as lactic acid bacteria were obtained at a low level as compared to the total viable count in the plates. A total of 126 isolates were obtained, out of which 100 isolates were from the oil spill sample and 26 isolates were from the Yak milk and the Yak churpi. All the strains were characterised for the presumptive tests of lactic acid bacteria then the strains were grouped according to cell morphology, gas production from glucose and ammonia production from arginine according to the positive and negative results.

About 45 isolates were screened for cholesterol utilization and among 45 isolates 13 showed significant cholesterol lowering property. These 13 strains were identified in the Biolog identification system. The bacteria from different genus like *Lactobacilli* and *Enterococcus* along with other genera like *Paenibacillus, Lysinibacillus, Gracibacillus* and *Dermabacter* were obtained. *Lactobacillus fructivorans* Y: CH7, *Dermabacter hominis* OL: C5 and *Enterococcus hirae* OL: C14, in particular have shown significant cholesterol lowering activity. The identified strains with significant cholesterol lowering activity was tested for probiotic property keeping in mind the three strains that had lower cholesterol lowering activity. *Enterococcus durans* (OL: CH4), *Lactobacillus fructivorans* (OL:

CH7, CH9,Yd), and *Enterococcus casseliflavus* CH10 and CH1 can be studied in future.

BIBLIOGRAPHY

Abdelgadir W.S, Hamad S.H, Møller PL, Jakobsen M. (2001). Characterisation of the dominant microbiota of Sudanese fermented milk Rob. *International Dairy Journal* 11:63–70.

Abidi S.L. (2001). Chromatographic analysis of plant s4terols in foods andvegetable oils, *Journal of Chromatography A* 935: 173–201.

Agerholm-Larsen L. (2000). Effect of 8 week intake of probiotic milk products on risk factors for cardiovascular diseases. *European Journal of Clinical Nutrition* 54(4):288-97.

Aguirre M and Collins M.D. (1993). Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology* 75:95–107.

Aiba Y., Suzuki N., Kabir A.M., Takagi A and Koga Y. (1998).Lactic acid-mediated supression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. *American Journal Gastroenterology* 93:2097–2101.

Allain C.C., Poon L.S., Chen C.G., Richmond W. and Paul C. Fu. (1974).Enzymatic determination of total serum cholesterol. *Clinical Chemistry*20: 470-475.

Ambalam P., Dave J.M., NairB.M and VyasB.R.M. (2011).*In vitro* Mutagen binding and antimutagenic activity of human *Lactobacillus rhamnosus. Anaerobe* 17(5):217-222.

ApostolidisE., KwonY. I., Rahul S, Reza G. and Shetty K.(2011).Inhibition of *Helicobacter pylori* by Fermented Milk and Soymilk Using Select Lactic Acid Bacteria and Link to Enrichment of Lactic Acid and Phenolic Content. *Food Biotechnology* 25(1):58-76.

Araki Y-I., Lee S., Sugihara G., Furuichi M., Yamashita S and Ohseto F.(1996).New cationic surfactants derived from bile acids: synthesis and thermodynamic and biophysicochemical properties such as membrane perturbation and protein solubilizing abilities. *Colloids and Surfaces B: Biointerfaces* 8:81–92.

Assmann G., Cullen P., Erbey J., Ramey D.R., Kannenberg F. and Schulte H. (2006). Plasma sitosterol elevations are associated with an increased incidence of coronary events in men: results of a nested case-control analysis of the Prospective Cardiovascular Münster (PROCAM) study. *Nutrition Metabolism Cardiovascular Diseases* 16:13–21.

Axelsson L. (1998). Lactic acid bacteria: classification and physiology. In: *Lactic acid bacteria Microbiology and Functional Aspects*, 2nd Edn., (Eds. Salminen, S. and Wright, A.V.)Marcel Dekker, New York. 1-72.

Badis A, Guetarni D, Moussa-Boudjemaa B, Henni DE, Tornadijo ME, Kihal M (2004). Identification of cultivable lactic acid bacteria isolated from Algerian raw goat's milk and evaluation of their technological properties. *Food Microbiology* 21:343–349.

Bazarre TL, Wu SL, Yuhas JA. (1983). Total and HDL cholesterol concentrations following yogurt and calcium supplementation. Nutr Rept Int 28:1225–1232.

Bazhenov L.G., Bondarenko V.M and Lykova E.A. (1997).The antagonostic action of lactobacilli on *Helicobacter pylori. Journal of Microbiology Epidemiology Immunology* 3:89–91.

Berkson DM, Stamler J, Lindberg HA, Miller W, Mathies H, et al. (1960). Socioeconomic correlates of atherosclerotic and hypertensive heart diseases. *Ann. N. Y. Acad. Sci.* 84:835–53.

Besselink M.G.,Van Santvoort H.C and Buskens. E. (2008).Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 371:651–659.

Beukes EM, Bester BH, Mostert JF. (2001). The microbiology of South African traditional fermented milks. *International Journal Food Microbiology*. 63:189–197.

Bodana A.S and RaoD.R.(1990).Antimutagenic Activity of Milk Fermented by *Streptococcus thermophilus* and *Lactobacillus bulgaricus. Journal of Dairy Science* 73(12):3379-3384.

Boehringer –Mannheim. (1989).UV method for the determination of L-lactic acid and D-lactic acid in foodstuffs and other materials. In: *Methods of Biochemical Analysis and Food Analysis using Single Reagents*. Boehringer-Mannheim GmbH, Biochemical: 78-81.

Bover-Cid S and Holzapfel W.H.(1999).Improved screening procedure for biogenic amine production by lactic acid bacteria .*International Journal of Food Microbiology* 53:33-41.

Brinkley A.W., Gotsmann A.R. and Mott G.E. (1980).Growth of Cholesterol-Reducing Eubacterium on Cholesterol-Brain Agar, *Applied and environmental Microbiology*: 1130-1132.

Buchanan.R.E. and Gibbons .N.E.(1974). *Bergey's Manual of Determinative Bacteriology*, 8th Edition. Williams and Wilkins: Baltimore.

Cabo M.L., Murado M.A., Gonzalez M.P. and Pastoriza L.(1999). A method for bacteriocin quantification .*Journal of Applied Microbiology* 87:907-941.

Cahn D. (1901). Bacilli of infant stools stainable according to Gram. *Centralbl. Bakteriol. eI. Abt. Orig* 30: 721–720.

Cebra J.J. (1999). Influences of microbiota on intestinal immune system development. *American Journal of Clinical Nutrition 69* : 1046S–1051S.

Chammas GI, Saliba R, Corrieu G, Be´al C (2006). Characterisation of lactic acid bacteria isolated from fermented milk ''laban''. Int J Food Microbiol 110:52–61.Cheeke P.R. (2000). Actual and potential applications of Yucca schidigera and Quillaja saponaria saponins in human and animal nutrition. *Journal of Anima Science* 77:1–10.

Collins J.K., Thornton G. and O'Sullivan G.O. (1998).Selection of probiotic strains for human applications. *International Dairy Journal* 8:487-490.

Conway P.L. (1996). Selection criteria for probiotic microorganisms. *Asia Pacific Journal of Clinical Nutrition* 5:10 –14.

Corzo G. and Gilliland S.E. (1999). Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus. Journal Dairy Science* 82:466–471.

Corzo G., Gilliland S.E. (1999). Measurement of bile salt hydrolase activity from *Lactobacillus acidophilus* based on disappearance of conjugated bile salts. *Journal of Dairy Science,* 82, 466-71.

Cotter P.D., Degan L.H.,Hill C and Ross P. (2004).Bacteriocins:Biological tools for biopreservation and shelf-life extension .4th NIZO Conference on Prospects for health ,wellbeing and Safety. *Elsevier science* Ltd, Rapendal Netherlands : 1058-1071.

Crowe C., Sanders C., Jr W. E and Longley S. (1973). Bacterial interference, Role of the normal throat flora in prevention of

colonization by group A *Streptococcus*. *Journal of Infection and Disease* 128:527–532.

Daneshfar A., Khezeli T and Lotfi H.J. (2009). Determination of cholesterol in food samples using dispersive liquid–liquid microextraction followed by HPLC–UV. *Journal of Chromatography B* 877: 456–460

Danielson A.D., Peo E.R., Shahani K.M., Lewis A.J., Whalen P.J. and Amer M.A.(1989). Anticholesteremic property of Lac*tobacillus acidophilus* yogurt fed to mature boars. *Journal of Animal Science* 67, 966.

Desobry-Banon S., Vetier and Hardy J. (1999). Health benefits of yogurt consumption.In: *International Journal of Food Properties* 2(1):1-12

Dewan S, Tamang J P, Leeuwenhoek A.(2007). Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. (92):343–352.

Dora I.A. and Glenn R. (2002). Cholesterol Assimilation by Lactic Acid Bacteria and Bifidobacteria Isolated from the Human Gut. *Applied and Environmental Microbiology.* 68 (9) 4689–4693.

Donohue D.C. and Salminen S.(1996). Safety of probiotic bacteria. *Asia Pacific Journal of Clinical Nutrition* 5:25-28.Durlu-Ozkaya F, Xanthopoulos V, Tunaı" N, Litopoulou- Tzanetaki E (2001). Technologically important properties of lactic acid bacteria isolated from Beyaz cheese made from raw ewes' milk. *Journal of Applied Microbiology* 91:861–870.

DuPont A. W. and DuPont H. L.(2011). *Nature Reviews Gastroenterology and Hepatology* 8.Dykes G.A., Britz T. J. and von Holy A.(1994). Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum packaged Vienna sausages. *Journal of Applied*

Bacteriology 76: 246-52.

Dunne C.L., Mahony M., Thornton G., Morrisey D., Hallorans S., Feeney M., Flynn S., Kiely B., Daly C., Collins K. (2001). *In vitro* selection criteria for probiotic bacteria of human origin: Correlation with *in vivo* findings. *AmericanJournal of Clinical Nutrition,* 73, 386-392.

Frick M., Elo O and Happa K. (1987). Helsinki Heart Study: Primary prevention trials with gemfibrogil in the middle-age men with dyslepimia. *England Journal of Medicine* 317: 237-245.

Friend B.A and Sahani K.M.(1984). Antitumor properties of Lactobacilli. *Journal of Food Proteins* 47:717-723.

Fukushima M. and Nakano M.(1996). Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* cholesterol metabolism in rats fed on a fat- and cholesterol enriched diet. *British Journal of Nutrition* 76: 857–867.

Gasser F. (1994). Safety of lactic acid bacteria and their occurrence in human clinical infections. *Bulletin de l'Institut Pasteur* 92:45–67.

Gibson G. R., Probert H. M., Rastall R. A and Roberfroid M. B.(2004). Dietary modulationof the human colonic microbiota: Updating the concept of prebiotics. Nutrition Research Reviews 17: 259–275.

Gibson G.R. (1998). Dietary modulation of the human gut microflora using probiotics. *Britain Journal of Nutrition,* 80: S209-S212.

Gill H.S. (1998).Stimulation of the Immune system by Lactic cultures .*International Dairy Journal* 8:535-544.

Gilliland S.E., Nelson C.R. and Maxwell C. (1985).Assimilation ofcholesterol by *Lactobacillus acidophilus. Applied Environmental Microbiology* 49: 377–381.

Gran HM, Gadaga HT, Narvhus JA. (2003). Utilisation of various starter cultures in the production of Amasi, a Zimbabwean naturally fermented raw milk product. *International Journal of Food Microbiology* 88:19–28.Gruenewald K.K. (1982). Serum cholesterol levels in rats fed milk fermented by *L. aciophilus. Journal of Food Science*47:2078 –2079.

Haenel H and Bendig J.(1975). Intestinal flora in health and disease. Progr. *Food Nutrition and science* 24:1-21.

Harrigan, W.F. (1998). *Laboratory Methods in Food Microbiology.*3rd edition. Academic Press London.

Havenaar R and Huis in't Veld J.H.J. (1992).Probiotics. a general view. In: The Lactic Acid Bacteria, *The Lactic Acid Bacteria In Health And Disease*, (EdsWood, B.J.B). Elsevier Applied Science, London 1 151–171.

Heilig H.G.H.J; Zoetendal E.G; Vaughan E.E; Marteau P; Akkermans A.D .L and de Vos, W.M. (2002). Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA, Appl. *Environmental Microbiology* 68:114.

Holt P.G. (1995). Environmental factors and primary T-cell sensitisation to inhalant allergens in infancy: reappraisal of the role of infections and air pollution. Pediatr. *Allergy Immunology* 6:1–10.

Holzapfel W.H., Haberer P., Snel J., Schillinger U. and Huis in't Veld J.H.J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology* 41:85-101.

Holzapfel, W.H. (1997). Use of starter cultures in fermentation on a household scale. *Food Control* 8 (5&6):241-258.

Holzapfel W.H., Giesen R. and Schillinger U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology* 24: 343-362.

Hongbao. M. (2004). Concept and Protocol to Isolate Cholesterol-reducing Bacteria from Carnivores.*Nature and Science* 2.

Hooper L.V., Midtvedt T. and Gordon J.I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annual Review Nutrition 22:283–307.

Horinaka M., Yoshida T., Kishi A., Akatani K, Yasuda T., Kouhara J ., Wakada M and Sakai T. (2011).*Lactobacillus*strains induce TRAIL production and facilitate natural killer activity against cancer cells. *FEMS Letters*584(3): 577- 582.

Hosono A. (2000). Effect of administration of Lactobacillus gasseri on serum lipids and fecal steroids in hypercholesterolemic rats.*Journal of Dairy Science.* 83(8):1705-11.

Huang J., Adams M.C. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy Propionibacteria. *International Journal of Food Microbiology.* 91, 253-260.

John S., Sorokin A.V and ThompsonP.D. (2007). Phytosterols and vascular disease. *Current Opinion of Lipidology* 18:35–40.

Jones D.(1978). Composition and differentiation of the genus Streptococcus. In: F.A Siknner and L.B Quesnel (editor), *Streptococci.* Academic après, London : 49.

Jones P.J.H, Howell T., Mac Dougall De.,Feng JY and Parsons W.(1998).Short –term administration of tall oil phytosterols improves plasma lipid profiles in subjects with different cholesterol levels. *Metabolism* 47:751-756.

Kabir A.M., Aiba Y., Takagi A., Kamiya S., Miwa T and Koga Y. (1997). Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut* 41:49–55.

Kandasamy M., Bay B-H., LeeY-K and Mahendran R. (2011). *Lactobacilli* secreting a tumor antigen and IL15 activates neutrophils and dendritic cells and generates cytotoxic T lymphocytes against cancer cells . *Cellular Immunology* 271(1): 89-96.

Katan MB ., Grundy SM., Jones P., Law M., Miettinen T and Pao H.(2003). Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clinic Proceedings* 78:965-78.

Kimoto H., Kurisaki J., Tsuji N. M., Ohmomo S., and Okamoto T. (1999).Lactococii as probiotic strains: adhesion to human enterocyte- like Caco-2 cells and tolerance to Low pH and bile. Letters.*Applied Microbiology*.29:313-316.

Klaenhammer T. R.,and Kullen M. J. (1999). Selection and design of probiotics. *International Journal of Food Microbiology* 50, 45–57.

Klein G., Pack A., Bonapare C., Reuter G.,Holzapfel W.H., Huis-in'-t-Veld J.H.J., Persin C and Kasper H. (1998). Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology* 41: 103-125.

Kurokawa K., Itoh T., Kuwahara T., Oshima K., Toh H. and Toyoda A.(2007). Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *DNA Res* 14:169–81.

Kramer W., Glombik H and Petry S. (2000). Identification of binding proteins for cholesterol absorption inhibitors as components of the intestinal cholesterol transporter. FEBS Letters 487:293- 7.

Laparra J.M and Sanz Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research* .61: 219–225.

Lawton E.M., Ross R.P and Hill C.(2007). Two-peptide lantibiotics: a medical perspective. *Mini Review of Medical Chemistry* 7: 1236 – 1247.

Leisner J.J., Rusul G., Wee B.W., Boo H.C. and Mohammad K. (1997) Microbiology of Chili Bo, a popular Malaysian food ingredient. *Journal of Food Protection* 60:1235-1240.

Li-Dong Guo., Li-jie Yang and Gui-Cheng Huo. (2011).Cholesterol removal by *Lactobacillus plantarum* isolated from homemade fermented Cream in inner Mongolia of China. *Czech. Journal of Food Science* 29(3):219-225.Lin S Y., Ayres J.W., Winkler W and Sandine W.E. (1989). Lact*obacillus* effects on cholesterol: *in vitro* and *in vivo* results. *Journal Dairy Review* 72:2885–2889.

Lindgren S.E.and Dobrogosz W.J(1990).Antagonistic activities of LAB in food and feed fermentations. FEMS Microbiology Reviews 87:149-164.

Liong M.T and Shah N.P. (2005). Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* strains. *Journal of Dairy Science*88: 55–66.

Luepker R.V. (2011). Cardiovascular Disease: Rise, Fall and Future Prospects.*Annual Review of Public Health* 32: 1-3.

Mackowiak P. A. (1982). The normal microbial flora. *New England Journal of Medicine* 307:83–93.

Marteau P., Gerhardt M.F., Myara A., Bouvier E., Trivin, F.,Rambaud, J.C.(1995). Metabolism of bile salts by alimentary bacteria during transit in the human small intestine. *Microbial Ecololgy Health and Disease* .8:151–157.

Marteau P., Pochart P., Dore J., Bera-Maillet C., Bernalier A and Corthier G.(2001). Comparative study of bacterial groups within the human cecal and fecal microbiota. *Applied Environmental Microbiology.* 67:4939.

Martin M.A., Pfaller M.A., Massanari R.M. and Wenzel R.P. (1989). Use of cellular hydrophobicity, slime production and species identification markers for the clinical significance of coagulase negative staphylococcal isolates. *American Journal of Infectious Control* .17: 130-135.

Matthan N.R., Pencina M., Larocque J.M., Jacques P.F., D'Agostino R.B., and Schaefer E.J.(2009). Alterations in cholesterol absorption and synthesis characterize Framingham offspring study participants with coronary heart disease. *Journal of Lipid Research* 50: 1927–35.

Matar C., Nadathur S.S., Bakalinsky A.T and Goulet J.(1997).Antimutagenic Effects of Milk Fermented by *Lactobacillus helveticus* L89 and a Protease-Deficient Derivative *Journal of Dairy Science*80 (9):1965-1970.

Mathara J. M., Schillinger U., Guigas C., Franz C., Kutima P.M., Mbugua S. K., Shin H.-K., Holzapfel W. H. (2008). Functional characteristics of*Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. *InternationalJournal of Food Microbiology,* 126, 57–64.

Mathara JM, Schillinger U, Kutima PM, Mbugua SK, Holzapfel WH (2004) Isolation, identification and characterization of the

dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. International Journal of Food Microbiology 94:269–278

Mann G.V. and Spoerry A. (1974). Studies of a surfactant and cholesteremia in the Massai.*American Journal of Clinical Nutrition* 27:464-469

Mel'nikov S.M, Seijen ten Hoorn J.W.M. and Eijkelenboom A.P.A.M. .(2004). Effect of phytosterols and phytostanols on the solubilization of cholesterol by dietary mixed micelles: an in vitro study. *Chemistry and Physics of Lipid* 127:121-141.

Metschnikoff E. (1907). The Prolongation of Life. In: *Optimistic Studies.* William Heinemann, London.

Metschnikoff E. (1908). Prolongation of Life. Putnam, New York.

Mercenier A., Pavan S and Pot B. (2003). Probiotics as biotherapeutic agents: present knowledge and future prospects. *Current Pharmaceutical Design* 9:175–191.

Miettinen TA., Puska P.,Gylling H., Vanhanen H and Vartiainen E.(1998).Reduction of sitistanol-ester margarine in a mildly hypocholesterolemic population. *New England Journal of medicine* 333:1308-1312.

Morelli L. (2000). In vitro selection of probiotic *Lactobacilli*: A critical appraisal. *Current Issues Intestinal Microbiology* 1: 59-67.

Moro E. (1900). *Bacillus acidophilus*: A contribution to the knowledge of the normal intestinal bacteria of infants. *Jahr*52: 38–55.

Mundt J.O. and Hammer, J.L. (1968).Lactobacilli On plant. Applied Microbiology. 16: 1326-1330.

Nagasawa M. and Bae I.(M. 1969) Microbial transformation of sterols. In:.Decomposition of cholesterol by microorganisms. *Journal of Agriculture and Biochemistry* 33:1644-1650.

Noh D.O., KimS. H and Gilliland S.E. (1997): Incorporation of cholesterol into cellular membrane of *Lactobacillus acidophilus* ATTC43121. *Journal of Dairy Science* 80:3107-3113.

Nostro A., Cannatelli M.A., Crisafi G., Musolino A.D., Procopio F. and Alonzon V. (2004). Modifications of hydrophobicity, *in vitro* adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Letters in Applied Microbiology* 38: 423-427.

Olasupo N.A., Shillinger U.and Holzpafel W.H. (2011). Studies on some technological properties of predominant lactic acid bacteria isolated from Nigerian fermented foods. *Food Biotechnology* 15(3): 157-167.

Ouwehand A. C., Kirjavainen P. V., Shortt C and Salminen S. (1999). Probiotics: Mechanisms and established effects. *International Dairy Journal* 9(1): 43–52.

Patel M.D. and Thompson P.D. (2006). Phytosterols and vascular disease. *Atherosclerosis* 186:12–9.Pelletier X., Laure-Boussuge S. and Donazzolo Y.(2001). Hydrogen excretion upon ingestion of dairy products in lactose intolerant male subjects: Importance of the live flora. *European. Journal of Clinical Nutrition* 55: 509–512.

Pelto L.,Isolauri E and Lilius E.M.(1998).Probiotic bacteria down regulate the milk-induced inflammatory response in milk hypersensitive subjects but have an immunostimulatory effect in healthy subjects. *Clinical Exp Allergy* 32:1474-1479.

Perez P.F., Minnaard Y., Disalvo E.A. and De Antoni G.L. (1998). Surface properties of bifidobacteria strains of human origin. *Applied and*

Environmental Microbiology 64: 21-26.

Pool-Zobel B.L., Munzner R and Holzapfel W.H. (1993). Antigenotoxic properties of lactic acid bacterium the *S. typhimurium* mutagenicity assay. *Nutrition and Cancer* 23:261–270.

Rani B., Khetarpaul N. (1998). Probiotic fermented food mixture: possible applications in clinical anti- diarrhoea usage. *Nutrition and Health* 12:97–105.

Rao D.R., Chawan C.B and Pulusani S.R. (1981). Influence of milk and thermophilus milk on plasma cholesterol levels and hepatic cholesterogenesis in rats. *Journal of Food Science* 46:1339–1341.

Rasic, J. L. (2003). Microflora of the intestine probiotics. In: Encyclopedia of food sciences and nutrition (Eds B. Caballero, L. Trugo, & P. Finglas)Oxford: Academic Press.pp:3911–3916.

Razin S., Kutner S., Efrati H., and Rottem S. (1980).Phosphplipid and Cholesterol uptake by mycoplasma cell and membranes. *Biochem .Biophys.acta* 598:628-640.

Reddy K. S., Shah B., Varghese C. and Ramadoss A. (2005). Responding to the threat of chronic diseases in India. *Lancet* 366:1744–1749.

Rizzello C.G., Nionelli L., Coda R and Gobbetti M.(2011). Synthesis of the Cancer Preventive Peptide Lunasin by Lactic Acid Bacteria During Sourdough Fermentation. *Nutrition and Cancer* 5:1-10.

Rosebury T. (1962). In:*Microorganisms Indigenous to Man*, McGraw Hill, New York.

Rosenberg M.(1984). Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiology Letters 22: 289-295.

Rogers P., Chen J and Zidwick M. (2006). Organic Acid and Solvent Production Part I: Acetic, Lactic, Gluconic, Succinic and Polyhydroxyalkanoic Acids. In: *The Prokaryotes*. (Editors. Dworkin M.,Falkow S., Rosenberg E and Stakebrandt E). Third Edition 1. Springer Science New York,USA. pp: 511-575.

Rudel' L.L. and Morris M.M . (1973). Determination of cholesterol using o-phthalaldehyde. *Journal of Lipid Research* 14:364-366.

Saikali J., Picard C., Freitas M and Holt P. (2004). Fecal Water Genotoxicity Is Predictive of Tumor-Preventive Activities by Inulin-Like Oligofructoses, Probiotics (*Lactobacillus rhamnosus and Bifidobacterium lactis*), and their Symbiotic Combination 49(2):144-155.

Saikali J., Picard C., Freitas M and Holt P. (2004). Fermented Milks,Probiotic Cultures, and Colon Cancer. *Nutrition and Cancer* 49(1):14-24.

Salminen. S.J. and Donohue D.C.(1996). Safety assessment of *Lactobacillus* strain GG (ATCC53103). *Nutrition Today* 31(suppl.): 12-15.

Salminen S., Isolauri E and Salminen E. (1996). Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Van Leeuwenhoek International. *Journal of General and Molecular Microbiology* 70:347–358.

Sandine WE., Radich P.C and Elliker P.R. (1972). Ecology of the lactic *Streptococci*.A review. *Journal of Milk Food Technology* 35:176-184.

Saran S., Gopalan S., Krishna T.P. (2002). Use of fermented foods to combat stunting and failure to thrive. *Nutrition* 18:393–396.

Schiffrin JE., Rochat F and Link-Amster H. (1994).Immunomodulation of Human Blood cells following ingestion of LAB. *Journal of Dairy Science* 78:491-497.

Schiller, D., Kruse, D., Kneifel, H., Kramer, R., Burkovski, A.,2000. Polyamine transport and role of potE in response to osmoticstress in Escherichia coli. Journal of Bacteriology 182,6247–6249.

Shillinger U and Lucke F.K.(1987). Identification of lactobacilli from meat and meat products. *FoodMicrobiology* 4: 199-208.

Silla Santos, M.H., 1996. Biogenic amines: their importance infoods. International Journal Food Microbiology 29, 213– 231.

Smith K., McCoy K. D. and Macpherson A. J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in Immunology.* 19:59–69.

Spanhaak S., R. Hanevaar, Schaafsma G. (1998). The effect of consumption of fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *European Journal of Clinical Nutrition.* 52:899–907.

Spilburg CA, Goldberg AC, McGill JB, Stenson WF, Racette SB, Bateman J, McPherson T, Ostlund RE.(2003) Fat-free foods supplemented with soy stanol-lecithin powder reduce cholesterol absorption and LDL cholesterol. *Journal of American Dietician Association*; 103: 577581.

Sudha R, Chauhan P, Dixit K, Babu K, Jamil K.. (2009). Probiotics as complementary therapy for hypercholesterolemia. *Review Article Biology and Medicine* Vol 1 (4): Rev4.

Sugano H. and Imaizumi K.(1986).Cholesterol: Sankyo Syuppan, Tokyo. pp: 122-145.

Suskovi B.K., Matošic S., Besendorfer V. (2000). *World J. Microbiol.*

Biotechnol. 16: 673–678.

Tamang J.P., Tamang B., Schillinger U., Guigas B., Holzapfel W.H. (2009). Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas. *International Journal of Food Microbiology* 135: 28–33.

Tamang J.P., Dewan S., Thapa S., Olasupo N. A., Schillinger U. and Holzapfel W. H. (2000). Identification and enzymatic profiles of predominant lactic acid bacteria isolated from soft-variety *chhurpi*, a traditional cheese typical of the Sikkim Himalayas. *Food Biotechnology* 14 (1&2):99-112.

Taranto M. (1999).Effect of *Lactobacillus reuteri* on the prevention of hypercholesterolemia in mice. *Journal of Dairy Science* 83:401-3.

Thornley M.J.(1960).The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. Journal of Applied Bacteriology.23:37-52.

Tichaczek P.S., Nissen – Meyer,J., Nes,I.F., Vogel,R.F. and Hammes,W.P.(1992).Characterization of the bacteriocin curvacin A from *Lactobacillus curvatus* LYH174 and sakacin P from *Lactobacillus sake* LHT637. *Systematic and Applied Microbiology* 15:460-468.

Tissier H.(1905). Repartition des microbes dans l'intestin du nourisson. *Annles de I'Instut Pasteur Paris*19: 109–123.Turpin W., Humbolt C.,Thomas M and Guyot JP.(2010).Lactobacilli as multifaceted probiotic with poorly disclosed molecular mechanisms. *International Journal of Food Microbiology*.143:87-102.

Usman and Hosono A. (1999).Bile tolerance, taurocholate deconjugation,and binding of cholesterol by *Lactobacillus gasseri* strains.*Journal of Dairy Science* 82:243-248.

SCH58235, in the rat and rhesus monkey through the identification of the active metabolites of SCH48461. *Journal of Pharmacology Experimental Therapeutics* 283:157- 63.

Vendt N., Grunberg H., Tuure T., Malminiemi O., Wuolijoki E., Tillmann V., Sepp E and Korpela R. (2006). Growth during the first 6 months of life in infants using formula enriched with *Lactobacillus rhamnosus* GG: double-blind, randomized trial. *Journal of Human Nutrition and Diet* 19:51–58.

Villena J.,Medina M and Vintinfii E.(2008).Stimulation of respiratory immunity by oral administration of *Lactococcus lactis.* Can Journal of Microbiology 54(8)630-638.

Von Mutius E; von Braun-Fahrla¨nder C; Schierl R ; Riedler J ; Ehlermann S; Maisch S ; Waser M and Nowak D.(2000). Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin. Exp. Allergy.* 30:1230–1234.

Walker C and Reamy B.V.(2009).Cardiovascular disease prevention:What is the evidence.*American Family Physican* 79(7):571-578.Wold A.E.(1998) The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 53 (suppl. 46): 20–25.

Xanthopoulos V, Petridis D, Tzanetakis N (2001) Characterization and classification of Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus strains isolated from traditional Greek yogurts. J Food Sci 66:747–752.

Zamfir M, Vancanneyt M, Makras L, Vaningelgem F, Lefebvre K, Pot B, Swings J, De Vuyst L (2006) Biodiversity of lactic acid bacteria in Romanian dairy products. Syst Appl Microbiol 29(6):487–495

SCH58235, in the rat and rhesus monkey through the identification of the active metabolites of SCH48461. *Journal of Pharmacology Experimental Therapeutics* 283:157- 63.

Vendt N., Grunberg H., Tuure T., Malminiemi O., Wuolijoki E., Tillmann V., Sepp E and Korpela R. (2006). Growth during the first 6 months of life in infants using formula enriched with *Lactobacillus rhamnosus* GG: double-blind, randomized trial. *Journal of Human Nutrition and Diet* 19:51–58.

Villena J.,Medina M and Vintinfii E.(2008).Stimulation of respiratory immunity by oral administration of *Lactococcus lactis.* Can Journal of Microbiology 54(8)630-638.

Von Mutius E; von Braun-Fahrla¨nder C; Schierl R ; Riedler J ; Ehlermann S; Maisch S ; Waser M and Nowak D.(2000). Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin. Exp. Allergy.* 30:1230–1234.

Walker C and Reamy B.V.(2009).Cardiovascular disease prevention:What is the evidence.*American Family Physican* 79(7):571-578.Wold A.E.(1998) The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 53 (suppl. 46): 20–25.

Xanthopoulos V, Petridis D, Tzanetakis N (2001) Characterization and classification of Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus strains isolated from traditional Greek yogurts. J Food Sci 66:747–752.

Zamfir M, Vancanneyt M, Makras L, Vaningelgem F, Lefebvre K, Pot B, Swings J, De Vuyst L (2006) Biodiversity of lactic acid bacteria in Romanian dairy products. Syst Appl Microbiol 29(6):487–495

Zubillaga M., Weill R. E., Goldman P., Caro C., Boccio R.(2001). Effect of probiotics and functional foods and their use in different diseases. *Journal of Nutrition Research* 21:569–579.

24. SOIL SCIENCE

SOIL AQUIFER TREATMENT

Umarfarooque Momin

Senior Research Fellow, Division of Resource Management, CRIDA Santoshnagar Hyderabad-500 059

So what is SAT and how does it fit into where our water comes from? Water on the earth is constantly on the move, recycling and being used over and over. This is called the water cycle. That means the water that we drink has also been used, disposed of, and used again in a never-ending cycle.

As part of the cycle, the earth soaks up some of the water and stores it in the ground until it is needed. This underground storage area is called an aquifer or groundwater basin. Some people pump from underground aquifers and use it for drinking water, household uses, or agricultural irrigation.

When water soaks into the ground it is purified naturally through physical, chemical and biological processes in the soil called soil aquifer treatment or SAT. This purification process has occurred since the beginning of time. Soil aquifer treatment enables water managers to replenish valuable groundwater resources and to ensure future water supplies for the growing population of the India.

Soil aquifer treatment has been used by people for more than 60 years in arid Arizona and Southern California to provide additional polishing of highly treated recycled water that is used to refill underground water supplies. It is an increasingly important water resource management tool as the regions continue to grow and experience droughts, and water becomes more precious.

SAT is similar to another process that has been used for centuries and is still in practice today, especially in Europe and other countries. There it is called "bank filtration," where river water filters through the banks and sides of rivers and moves into groundwater basins.

FIG. 1: process of soil natural purification of water

25. HORTICULTURE

LARGE CARDAMOM: THE QUEEN OF SPICES

Kriti Ghatani

Department of Microbiology, School of Life sciences, Sikkim University, 6th Mile, Samdur, Tadong, Gangtok -737102

Introduction: Large Cardamom or black Cardamom (*Amom subulatum*) is regarded as the "Queen of spices" belongs to the family Zingiberaceae and order Sitaminae. Large Cardamom is called Greater Indian cardamom and its vernacular is *Bada Elaichi* in Hindi and *Thulo Elaichi* in Nepali. India is the largest producer and an exporter of spice accounting for about 60% of the global market. The demand for large cardamom in the export market is increasing steadily. Black cardamom is grown in the Sub Himalayan region in the North eastern states and in parts of Uttaranchal 0.88% of large cardamom is obtained from Sikkim and Darjeeling hills. The fruit of the plant that forms the capsule contains a lot of moisture and can be echinated by rubbing after curing.

Large Cardamom have long been used as an ayurvedic medicine and a spice as it is highly aromatic. It is used as a flavouring agent in foods.

Cultivation: Guatemala, India, Srilanka, Tanzania, El Salvador, Vietnam, Laos, Cambodia and Papua New Guinea are the major cardamom

growing countries. Large cardamom cultivation in India originally started in the Eastern Himalayas particularly in the Sikkim and the Darjeeling hills. Its cultivation began around 50 years ago in district in East Nepal.

Botanical Description: Large Cardamom is a perennial herb growing 1.8-2.5 m tall, with leafy shoots and panicles. The leafy shoots are grayish or reddish and cylindrical depending on the variety. Leaves are unicosate with parallel venation.

Medicinal benefits of large cardamom

It is used as a spice and in several ayurvedic preparations and in aroma therapy.

As a diuretic: It helps the body to release excessive fluid out of the body, and aid in treating of sciatica, kidney stone, lymphatic swelling, gonorrhea, liver disorders and high blood pressure.

As a carminative: It prevents the formation of gas in the gastrointestinal tract.

As a stomachic: In stimulating gastric digestion and sharpening appetite.

As a cardiac stimulant: Curcumin is a potent antioxidant in cardamom. The antioxidant protects the heart by preventing the deposition of cholesterol from narrowing the arteries, called atherosclerosis which is the main cause of heart attacks.

As an antiemetic: It is used combat nausea and vomiting, often useful for migraine.

An antidote for snake and scorpion venoms: An antidote is a substance that can counteract the effect of poisoning. Many healers in South Asia have been successfully using this herb for centuries as an antidote for snake venom and scorpion. Modern researches have yet to prove these facts

For respiratory troubles: It is also used as remedy for throat and respiratory troubles. It is also reported that large cardamom seeds are used as preventive as well as curative measure for throat troubles, congestion of lungs, and in the treatment of pulmonary tuberculosis

The decoction of seeds is used as a gargle in infection of teeth and gums.

Antiseptic property: It contains an essential oil called cineole: Cineole is an antiseptic that can kill bad breath bacteria, which may explain in part its reputation as an aphrodisiac. Cineole is often used by aroma therapists as a remedy for fainting and it is believed to attack the central nervous system.

The demand for large cardamom in the export market is increasing steadily. There has been a surplus export of large cardamom over the decades. But there has been a continuous decline due to natural calamities such as draught, hailstorm, snowfall, widespread occurrence of viral diseases and recently fungal diseases. Major problem due to viral diseases are most common. Development of anti-viral agents will help the nation to increase the commercial productivity.

26. HORTICULTURE

HIGH DENSITY PLANTING IN FRUITS CROPS

P. G. Naik, D. J. Sanap, V. K. Patil and A. P. Naik

Ph.D. Scholars, College of Agriculture, Marathwada Agriculture University, Parbhani. (M.S.)

The conceptual background of High Density Planting (HDP) reveals that it was pioneered for emperate fruits in Europe. High density orchards ere first planted in Europe at the end of the ineteen sixties and since then there is a decline in aditional orchards with low densities. The exact mits of plant density be termed as high density is ot yet well defined. It varies with the region, ecies, variety, rootstock, cost of planting material, bour and the likely return from the orchard, and ro-techniques adopted for a particular crop.

High density planting is defined as planting at a nsity in excess of that which auffices to give aximum crop yield at maturity if the individual e grows to its full natural size. In other words it is planting of more number of plants than timum through manipulation of tree size.

HDP is one of the improved production hnologies to achieve the objective of enhanced productivity of Indian Fruit Industry. Yield and quality of the produce are two essential components of the productivity. HDP aims to achieve the twin requisites of productivity by maintaining a balance between vegetative and reproductive load without impairing the plant health.

The underlying principle of High density planting is to make the best use of vertical and horizontal space per unit time and to harness maximum possible return per unit of inputs and national resources. In India the usefulness / vitality of this technology has been proved in an array of fruit crops e.g. Pineapple, Banana, Papaya, Mango, Apple and Citrus.

Advantages: It induces precocity, increases yield, improves fruit quality and reduces labour cost resulting in low cost of production. it also enables the mechanization of fruit crop production and