# A Study on Cholesterol Lowering Property of Lactic Acid Bacteria from Yak Milk Products and Healthy Human

**A Thesis Submitted**

To

# Sikkim University



**In Partial Fulfilment of the Requirement for the**

## Degree of Doctor of Philosophy

By

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## June 2018

Dedicated
To
My Beloved Aama,
Baba & Bunu

# CERTIFICATE

This is to certify that the PhD thesis entitled **"A Study on Cholesterol Lowering Property of Lactic Acid Bacteria from Yak Milk Products and Healthy Human"** submitted to the **SIKKIM UNIVERSITY** in partial fulfilment for the requirement of the Doctor of Philosophy in Microbiology, embodies the work carried out by **Kriti Ghatani** for the award of Ph D. Degree in Microbiology, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. It is a record of bonafide investigation carried out and completed by her under my supervision and guidance. She has followed the rules and regulations laid down the University. The results are original and have not been submitted anywhere else for any other degree or diploma.

It is recommended this PhD thesis to be placed before the Examiners for evaluation.


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# DECLARATION

I declare that the present Ph.D thesis entitled **"A Study on Cholesterol Lowering Property of Lactic Acid Bacteria from Yak Milk Products and Healthy Human"** submitted by me for the award of the degree of **Doctor of Philosophy in Microbiology** of Sikkim University under the supervision of **Dr. Buddhiman Tamang,** Assistant Professor, Department of Microbiology, School of Life Sciences, Sikkim University,  is my original research work solely carried out by me in  the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok. No part thereof has been submitted for any degree or diploma in any University/ Institution.

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## Abbreviations

| | | | |
|---|---|---|---|
| % | Percentage | GIT | Gastrointestinal Tract |
| < | Less than | GDCA | Glycodeoxycholate |
| > | Greater than | GRAS | Generally regarded as safe |
| ≤ | Less than equal to | IHD | Ischemic Heart Disease |
| μg | Microgram | LAB | Lactic Acid Bacteria |
| μl | Microliter | LCA | Lithocholic acid |
| μm | Micrometer | LDL | Low-Density Lipoproteins |
| μM | Micromolar | HDL | High-Density Lipoprotein |
| (G+C%) | Gramine + Cytosine Percentage | HFD | High Fat Diet |
| ml | Millilitre | MIC | Minimum Inhibitory Concentration |
| BSH | Bile Salt Hydrolase | NCEP | National Cholesterol Education Program |
| CA | Cholic Acid | Ntn | N-terminal Nucleophilic hydrolases |
| CDCA | Chenodeoxycholic Acid | KOH | Potassium Hydroxide |
| CLSI | Clinical and Laboratory Standards Institute | RHD | Rheumatic Heart Disease |
| CVD | Cardiovascular disease | SG | Sodium Glychocolate |
| CSH | Cell Surface Hydrophobicity | ST | SodiumTaurocholate |
| CHD | Coronary Heart Disease | TDCA | Taurodeoxycholic acid |
| DCA | Deoxycholic Acid | TG | Triglycerides |
| EME | Established Market Economies | TC | Total Cholesterol |
| FAO | Food and Agriculture Organisation of the United Nations | V-LDL | Very Low Density Lipoprotein |
| GBD | Global Burden of Disease | WHO | World Health Organisation |
| GI | Gastrointestinal | | |

# Contents

# Introduction

# 1. Introduction

Cardiovascular disease (CVD) is one of the most important concerns accounting for one-third of all deaths in the world alone (Benjamin et al., 2017; Roth et al., 2017). The Global Burden of Disease (GBD), 2015 study conducted on disease incidence, prevalence, mortality and up-to-date estimates for cardiovascular burden confirmed an estimated 422.7 million cases of CVD and 17.92 million CVD deaths (Roth et al., 2015). The global number of deaths from CVD has increased during the past decade by 12.5% (GBD, 2015) due to population growth and aging mostly occurring in countries of South and East Asia because of their huge and rising populations (Joseph et al., 2017). Between 2005 and 2015 the death rates have declined by 15.6% according to age-specificity although recent data of 2017 suggest that the rate of decline has been slowing (Norheim et al., 2015; Roth et al., 2017). It is reported that declines have been greatest in high-income countries and are also occurr in many middle and low-income countries (Roth et al., 2015; Norheim et al., 2015). CVD deaths are on account to ischemic heart disease (IHD), stroke, hypertensive heart disease leading in heart failure, cardiomyopathy, rheumatic heart disease (RHD), and atrial fibrillation (Roth et al., 2015; Centre for Disease Control and Prevention Report, 2015). The elevation of serum cholesterol is considered to be the main reason for CVD (Brown & Goldstein, 1984; Khedkar et al., 1993; Chapman et al., 2011; Vos et al., 2015). In blood, the normal cholesterol level is < 200 mg/dl, borderline-high level is 200-239 mg/dl and > 240 mg/dl is considered high level. Higher than 1 mmol of cholesterol (ie. 18mg/dl) than the normal level is reported to increase the coronary heart disease and coronary death.

Majority of cholesterol in humans is synthesized in the liver and 25% synthesis occurs in the cells of the intestine where the additional sources of intestinal cholesterol come from the diet or are reabsorbed from bile (Tomkin et al., 2012). Cholesterol in food upon consumption is absorbed in the small intestine and some of the cholesterols are conjugated to glycine and taurine bile salts or into phospholipids to form micelle (Araki et al., 1996). Serum cholesterol include the transporters like high plasma concentrations of low-density lipoproteins (LDL) and triglycerides (TG) and low high-density lipoprotein (HDL) levels are risk factors for cardiovascular disease (CVD) according to National Cholesterol Education Program (NCEP), (Rezaianzadeh et al., 2012). Several studies and clinical trials have shown a link between cholesterol elevation and cardiovascular diseases (Stamler et al., 2000). The cholesterol accumulation in the arteries is reported to cause plaque formation thereby blocking the flow of blood from the heart ventricles and to the rest part of the body. High concentrations of low density lipoprotein (LDL) promote atheroma development in arteries also known as atherosclerosis (Tomkin et al., 2012).

Cholesterol lowering statins are expensive and are known to cause severe side effects (Golomb & Evans, 2008). Hence there is a growing public attention to the healthy food due to an increasing knowledge in society. Research in the field of probiotics has been increasing with its demand and there has been immense progress on the isolation, selection and characterization of specific cultures that have probiotic properties infused within them on promoting health benefits. Thus, a probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance. According to Food and Agriculture Organization of United Nations (FAO)/World Health Organisations (WHO), 2014 and an updated consensus statement by the International Scientific Association for

Probiotics and Prebiotics, a probiotic is defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). However, in the food industry, it is described as "live microbial food ingredients that are beneficial to health" (Clancy, 2003). The main criterion for a probiotic bacterium is to tolerate the action of lysozyme present in the oral cavity (Fuller, 1992), and then the stomach and upper intestinal tract where it is exposed to low and high pH conditions, bile acid toxicity, the stress conditions in the colon (Gibson et al., 2000) and it's hydrophobic nature to mediate adhesion (Otero et al., 2004).

A number of health benefits of probiotic bacteria have been recognized till date, infectious diseases of the gastrointestinal tract, atopic eczema, urogenital and respiratory diseases, diarrheal diseases, respiratory infections particularly common cold, flu and pneumonia, inflammatory bowel disease including chron's disease, ulcerative colitis, pouchitis, cancer types like colon cancer, bladder cancer, diabetes, allergies and diseases related to high cholesterol potentials have also been recognized (Bernet Camard et al., 1997; Isolauri et al., 2000; Reid & Bruce 2001; Levy, 2000; Gupta et al., 2000; Gopal et al., 2001; Hatakka et al., 2001; Ohashi et al., 2002; Matsuzaki et al., 2007; Taylor & Mitchell 2007; Kandasamy et al., 2011; Lewis et al., 2005; Guan et al., 2017). The increasing evidences that prove numerous health benefits of probiotics are shown in Figure 1.

These probiotic bacteria mostly belong to LAB and Bifidobacteria along with multiple other genera like Bacilli, Streptococci, Clostridia, yeasts and fungi (Neish, 2009). Probiotics are mainly dairy based supplied in the form of fermented milk and yoghurt or as of lyophilized powders, tablets, capsules, sprays or pastes depending on

the animal or human receiving the supplement and the condition to be treated. These capsules and tablets are not used for medical applications but used as health
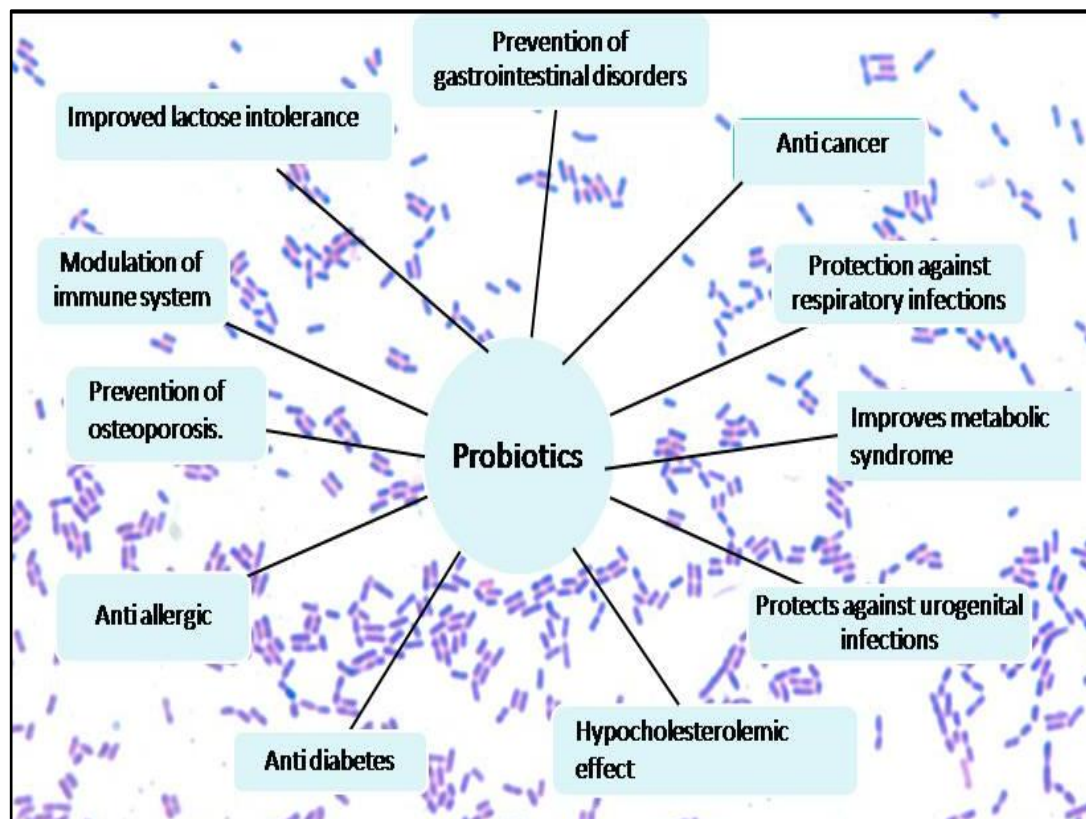


**Figure 1:** Health benefits of Probiotics

maintaining agents thereby producing a protective or beneficial effect on the gut flora of the host.Lactic acid bacteria (LAB) are also known as lactics, they are Gram-positive, catalase negative, non-spore forming, non-respiring cocci or rods, microaerophilic and produce lactic acid when carbohydrates are broken down (Axelsson, 2004; Klein et al., 1998). LAB particularly lactobacilli, streptococci enterococci and bifidobacteria are important microbiota present in the human gastrointestinal (GI) tract (Haenel et al., 1975; Marteau et al., 2001). LABs are used as starter culture for fermentation of meat and milk production. They are known to contribute to the stabilizing the balance of host intestinal microbiota and generally regarded as safe (GRAS) after safety evaluation tests through scientific procedures

(Bhattacharaya & Das, 2010). Lactics include different genera viz., *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus, Weissella* and *Sporolactobacillus* (Axelsson, 2004; Horvath et al., 2009; Thamacharoensuk et al., 2015). LAB are found in decomposing plants, animal matter soil, various fermented foods such as fermented milk, curd, yoghurt, cheese, whey, butter, buttermilk, bread, pickle, wine and fermented sausages (Rhee et al., 2011).

Several investigations on cholesterol lowering strains of probiotic bacteria have been conducted from the rich fermented foods and milk products in various parts of the world. *In vitro* and *in vivo* studies have identified hypercholesterolaemic probiotics strains belonging to *Lactobacillus* sp., *Pediococcus* sp., *Lactococcus* sp., *Enterococcus* sp. and *Streptococcus* sp. Some cholesterol lowering probiotic LAB strains that have been reported are as follows; *Lactobacillus acidophilus* (Lewis et al., 2005; Rajkumar et al., 2014), *Lactobacillus acidophilus* CHO-220 (Ooi et al., 2010), *Lactobacillus reuteri* NCIMB 30242 (Jones et al., 2012), *Lactobacillus bulgaricus* A7(LA7) (Bukowska et al., 1998; Madani et al., 2013; Rajkumar et al., 2014), *Lactobacillus bulgaricus* HLX37( Guan et al., 2017), *Pediococcus pentosaceus* KID7 (Damodharan et al., 2015), *Lactococcus lactis* NCDO 2118 (Oliveira et al., 2017), *Enterococcus faecium* (Agerholm et al., 2000), *Enterococcus faecium* CRL 183 (Cheik et al., 2008) etc.

*L. plantarum* and *L. johnsonii* (Pinto et al., 2006), *Lactobacillus rhamnosus* and *Lactobacillus paracasei* (Verdenelli et al., 2009), *Lactobacillus fermentum* LAB8, *L. mucosae* LAB12, *L. rhamnosus* LAB11, *L. salivarius* LAB9 and

*Weissella confusa* LAB10 (Beasley et al., 2006) were some strains possessing probiotic property that were reported from human feces.

The mechanisms of cholesterol-lowering action of probiotic bacteria in several *in vitro* studies have been proposed (Gilliland et al, 1985; Klaver & Van der Meer 1993; Tahri, 1995; Noh, 1997; Hosono, 1999; Lin Chen, 2000; Chiu et al., 2006; Kumar et al., 2011) although the exact mechanism of cholesterol lowering is poorly understood and the hypotheses for mechanism need to be confirmed in animal and human studies. The proposed mechanism of cholesterol by probiotics include binding of cholesterol to the cellular membrane (Noh, 1997), assimilation of cholesterol by the growing cells (Grill et al., 2000; Pereira & Gibson, 2002; Ziarno et al., 2007), cholesterol incorporation into the cellular membrane (Kimoto et al., 2002), bile salt hydrolase (BSH) or products containing them through interaction with host metabolism of bile salt (Smet et al., 1995), deconjugation of bile salts by the action of bile salt hydrolase enzyme (Liong & Shah, 2005), co-precipitation of cholesterol with deconjugated bile (Corzo & Gilliland, 1999; Pereira & Gibson, 2002) and conversion of cholesterol to coprostanol (Sadzikowski et al., 1977; Freier et al., 1994; Kumar et al., 2012).

In the present study, two different samples; human feces and traditional fermented yak milk products were used for investigating cholesterol lowering nature, then their probiotic characteristics and the possible mechanisms of cholesterol lowering. Although LAB is the one of less dominant microflora of fecal sample (Sghir et al., 2000) they also possess enzymatic and fundamental role to benefit the host physiology, therefore human fecal sample was chosen as one of the source. As there have also been reports of LAB strains being isolated from human gastrointestinal tract

(GIT) that may live commensally and adapt to intestinal environment (Ahrne et al., 1998).

Although the cholesterol-lowering ability of probiotics and probiotic-based dairy foods has been investigated by different groups across the world, the cholesterol lowering function of LAB strains from indigenous fermented food and milk product of yak has not been investigated from this region. Hence the present study has been focussed in the properties of LAB emphasising on its cholesterol lowering effect and its objective of using them for the management of high cholesterol.

The study was aimed at achieving the following objectives:

1. Isolation and preliminary characterization of lactic acid bacteria (LAB).

2. To screen the bacterial isolates for cholesterol lowering.

3. To study additional probiotic properties (acid tolerance, bile tolerance and hydrophobic nature) of LAB.

4. To study the mechanisms of cholesterol removal of the LAB isolates.

   - To screen the cultures for Bile salt hydrolase (BSH) activity.

   - To determine quantitative BSH activity.

   - To study bile acid deconjugation activity and co- precipitation of cholesterol.

   - To study the incorporation of cholesterol into the cellular membrane.

   - To study the assimilation of cholesterol in presence of carbohydrates and lipid containing media.

5. To identify the isolates having cholesterol lowering and probiotic properties by phenotypic method (sugar fermentation).

6. Confirmation of identity of isolates by genotypic method.

# Review

# of Literature

## 2. Literature Review

### 2.1. Problems related to high cholesterol

One of the most important concerns in the world today is the death due to cardiovascular diseases (CVD) (Benjamin et al., 2017; Roth et al., 2017). In the United States, CVD is listed as the elementary cause of death, accounting for nearly 836, 546 deaths, about 1 of every 3 deaths (Benjamin et al., 2018). Coronary Heart Disease (CHD) is the chief cause of deaths attributing to cardiovascular disease (43.8%), followed by stroke (16.8%), heart failure (9.0%), high blood pressure (9.4%), diseases of the arteries (3.1%), and other heart diseases (17.9%) (Benjamin et al., 2018). The number of persons dying from heart disease raised from 0.62 million in 1990 to 0.78 million in 1995, and 1.13 million in 2010 (Global Burden of Disease Study (GBD), 2013). There were prevalent cases of an estimated 422.7 million of CVD in 2015, according to the GBD 2015 (Roth et al, 2017). According to Heart Disease and Stroke Statistics Update 2015, cardiovascular disease, one of the leading causes of global death accounts for 17.3 million deaths per year and it is expected to grow to more than 23.6 million by 2030 in number (Mozaffarain et al., 2015). As per the WHO bulletin 2013, cardiovascular diseases and cancers are said to be the leading causes of death being responsible for 46% and 14% respectively of total deaths in women over 50, in 2050.

As estimated by the Global Burden of Disease study, in India the age-standardized CVD death rate is 272 per 1 lakh population which s higher than the global average of 235 per 1 lakh population (Prabhakaran et al., 2016). The evolution of the epidemic is defined by the alteration of socioeconomic levels that include that include; tobacco, unhealthy diets, such as those high in fat, salt, and free sugar and low in complex carbohydrates, and less consumption of fruit and vegetable that has

become more widespread among those from low socioeconomic backgrounds. The Indian population from these low socioeconomic backgrounds also do not receive the best possible therapy (Prabhakaran et al., 2016). A recent study reported that Hypercholesterolemia (total blood cholesterol of ≥200 mg/dl concentration) is present in 25–30% of urban and 15–20% rural population in India (Gupta et al., 2017).

Death due to CVD is likely to occur in young and middle aged individuals (30-69 years). The study with established market economies (EME) showed Indians currently experience CVD deaths at least a decade earlier than other countries. In the Western populations 23% of CVD deaths occur before the age of 70 years whereas in India, this number is 52% (Harikrishnan et al., 2014). The increase in levels of certain blood lipids has been reported to be the principal cause of cardiovascular disease and the leading cause of death and disabilities in developed countries (Bhatnagar et al., 2015).

Absorbed cholesterol contributes to increased risk of CVD (Brown & Goldstein, 2009; Assmann, 2006; Matthan et al., 2009; Chapman et al., 2011; Moss et al., 2016) specifically coronary heart disease; stroke, congestive heart failure, and peripheral artery disease cause death (Walker & Remy, 2009). The increase in levels of certain blood lipids have been reported to be the principal cause of cardiovascular disease and the leading cause of death and disabilities in developed countries (Bhatnagar et al., 2015). Low density lipoprotein (LDL) contributes as one of the major risk factors for coronary heart disease (CHD) as reported by expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (National Cholesterol Education Program (NCEP), 2001). It was reported that the decrease in LDL-cholesterol of 1 mmol/l concentration was linked with a 2.8 fold casual risk of

coronary death (Varbo et al., 2013). Similarly, 10% reduction in total cholesterol would reduce the risk of CHD by 20% (Probstfield & Rifkind, 1991).

According to WHO the risk of heart attack is three times higher in those with hypercholesterolemia, compared to those who have normal blood lipid profiles. 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). Methods or ways for lowering blood cholesterol levels involve dietary management, regular exercise, and drug therapy (Dunne, 2001).

## 2.2. Cholesterol metabolism

The word Cholesterol comes from the Greek word *chole-* (bile) and *stereos* (solid) followed by the chemical suffix -ol for an alcohol, it is an organic molecule. Daily intake of cholesterol regulates the level of cholesterol synthesis. 150 - 200 mg/dl of cholesterol is maintained in the body and controlled by the de novo synthesis (Thomas et al., 2012). About 1 g cholesterol is synthesized per day in healthy adults out of which 0.3 g of cholesterol is excreted (Thomas et al., 2012). Enough cholesterol is synthesized in normal healthy human body, which means dietary cholesterol intake is normally not required (Tabas, 2002). While major amount of cholesterol is used up in the synthesis of bile acids (Stamp & Jenkins, 2008) remaining cholesterol are utilized for biosynthesis of steroid hormones and vitamins (Payne & Hales, 2004), incorporation into the cellular membranes (Krause & Regen, 2014) etc.

Bile is a yellow-green water-soluble steroid end product of cholesterol produced in pericentral hepatocytes of the liver, mainly composed of bile salts (Carey et al., 1994; Hofmann, 1994). It is stored and concentrated in the gallbladder and

released into the duodenum through the bile duct upon ingestion of food (Johnson, 1998; Begley et al., 2006).  Other components of bile acids include phospholipids, glycine and taurine conjugates of bile acids that act as natural ionic detergents, bile pigment biliverdin and electrolytes (De Aguiar Vallim et al., 2013; Appleby & Walters, 2014; Camilleri & Gores, 2015). The detergent property of bile gives strong antimicrobial activity, primarily through the dissolution of bacterial membranes (Begely et al., 2005). Bile salts play an important role in the intestine in emulsifying lipids, which facilitate intra-luminal lipolysis and absorption of the lipolytic products by the enterocytes.

Bile acids are surfactants or detergents that promote absorption or emulsification of fats in the small intestine (Insull, 2006; Lefebvre et al., 2009). They have also been recognised to play a role as hormones in the regulation of metabolic processes (Houten et al., 2006). There are two major bile acid biosynthetic pathways in the liver, the neutral and alternative pathway (Vlahcevic et al., 1996). The neutral bile acid biosynthetic pathway consists of at least 16 enzymatic steps leading to the formation of cholic acid and chenodeoxycholic acid. The ratio of cholic acid to chenodeoxycholic acid in this pathway is controlled by sterol 12α hydroxylase (CYP8B1) (Russell, 2003). The primary bile acids are cholic acid (CA; 3α, 7 α , 12 α -.trihydroxy-5β-cholan-24-oic acid) (Kurdi et al., 2003) and chenodeoxycholic acid (CDCA; 3α , 7 α -dihydroxy-5 β -cholan-24-oic acid). These primary bile acids are conjugated either with glycine or taurine (Hofmann, 1999). Further the primary bile acids may undergo deconjugation by the bacterial enzymes called bile salt hydrolase (BSH). Other primary bile salts may undergo dehydroxylation at the 7th carbon atom to form deoxycholic acid (DCA) from CA or lithocholic acid (LCA) from CDCA (Stamp & Jenkins, 2008).

The neutral pathway of bile acid biosynthesis appears to be the major pathway of bile acid synthesis in humans under normal conditions. However, during various pathophysiological conditions, the alternative pathway of bile acid synthesis is the most active (Axelson & Sjovall, 1990). This pathway of bile acid biosynthesis is started by mitochondrial sterol 27-hydroxylase (CYP27A1). The liver and intestine are the sites of cholesterol synthesis and metabolism. A unique aspect of the intestine is the presence of a large and diverse bacterial population, which impacts greatly on this process (Feild, 1990). In humans cholesterol synthesized by the intestinal cells is introduced into the small intestine via exfoliation of intestinal cells. Additional sources of intestinal cholesterol come from secreted bile and the diet. Food cholesterol ingested is absorbed in the small intestine and some of the cholesterols are conjugated with glycine, taurine, bile salts, phospholipids to form micelle (Araki et al., 1996). In many animal species mucosal cells secrete cholesterol directly into the lumen (Lutton, 1976) however; this does not appear to occur in humans (Spritz, 1965). Cholesterol is excreted through the feces in humans and mammals. After meal gallbladder contraction causes bile to be released into the duodenum thus entering the enterohepatic circulation. Deposition of cholesterol in the bloodstream can form blockages in arterial walls thereby causing the arteries to become thicker, harder and less flexible, slowing down or/and sometimes blocking blood flow to the heart and other vital organs. The blockage results in angina (chest pain). A heart attack occurs when blood flow to the heart is severely impaired and a clot stops blood flow completely. High concentrations of LDL promote atheroma development in arteries (arteriosclerosis). But high concentrations of HDL can remove cholesterol from cells and prevent atheroma formation. However, these balances are genetically determined

and can be changed by body fluids, medications, food choices and other factors (Durrington, 2003).

Earlier studies have shown that complete elimination of dietary cholesterol and eliminating fat content to < 10% of the daily caloric intake together with stress management and aerobic exercise, results in only a 4% deterioration of atherosclerotic plaques after 5 years (Ornish et al., 1990).

Number of pharmacological agents is currently available for the treatment of elevated LDL-C. These include statins (3-hydroxy-3-methylglutaryl coenzyme, reductase inhibitors), fibrates (fibric acids), niacin (nicotinic acids), cholesterol absorption inhibitors, and bile acid sequestrants. Due to a long history of safety and efficacy, statins are the foundation stone of lipid-lowering therapy for reducing the levels of LDL-C (Brautbar & Ballantyne, 2011). Pharmaceutical drugs like Lipitor, Crestor, Zocor, Pravachol, Lovastatin, etc. are known to effectively reduce cholesterol levels; however, they are expensive and are known to have severe side effects (Bliznakov, 2002). Statins are reported to reduce CVD-related events by approximately 25% (Baigent et al., 2005). Although statin therapy has been shown to reduce the incidence of first or persistent coronary artery disease related events but the majority of patients with high risk fail to respond (Andrews et al., 2001; Foley et al. 2003; Ford et al., 2003; Blaha et al., 2012). In cases, statin therapy probably lead to the adverse effects related to the use of this class of drugs, which include myopathy (Redberg et al., 2017) and cognitive impairment (Muldoon et al., 2000; Muldoon et al., 2004; Fernandez, 2011). However, studies revealed doubling of the statin dose resulted in only a 6-8% LDL-C reduction using currently available treatments (Jones et al., 1998). They have also been established to be less effective in lowering other markers, such as triglycerides and lipoproteins that are associated with the risk of

atherosclerosis (Sarwar et al., 2007; Bennet et al. 2008). The ineffectiveness of cholesterol lowering medicines was reported in several clinical trials with primary and secondary prevention where the risk of CVD had increased continuously to 30% during the treatment after discontinuing medication for 5 years (Insull, 1997). Non-pharmacological treatment serves as a supportive therapy to reduce cardiovascular risk in otherwise healthy people.

## 2.3. Lactic acid bacteria

The first pure culture *Bacterium lactis* (now probably *Lactococcus lactis*) was obtained by Joseph Lister in 1873 (Santer, 2010). Lactic acid bacteria (LAB) are diverse and ubiquitous species of Gram-positive, catalase negative, non-sporeforming, microaerophilic cocci or rods and commonly produce lactic acid when carbohydrates are metabolized (Axelsson, 2004; Klein et al., 1998). They obtain ATP by fermentation of sugars as they are unable to synthesize cytochromes and porphyrins for ATP generation. They can grow under anaerobic conditions and in the presence of oxygen; hence LABs are called aero tolerant anaerobes (Stieglmeier et al., 2009). According to the classification system provided by Orla-Jensen to the genus level first divided LAB according to morphology as rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera) as cited in Pertterson et al. (1985). Then LAB was characterized according the mode of glucose fermentation as homofermentative and heterofermentative bacteria. Homofermentative LAB are those that convert sugars to lactic acid by glycolysis (Embden-Meyerhof pathway) (Abbott et al., 2009) and heterofermentative bacteria produce not only lactic acid but ethanol/acetic acid, and carbon-dioxide. LAB species belong to two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes, the order Lactobacillales include the following

genera: *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus and Weissella*, which have a low guanine cytosine (G+C%) percentage of 31–49% content organisms (Horvath et al., 2009). Another genus *Sporolactobacillus* (Thamacharoensuk et al., 2015) has been recently incorporated. However, in the Actinobacteria phylum there are two genera, the *Atopobium* and *Bifidobacterium* that 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 %). These two genera of Actinobacteria have a GC content of 36-46% and 58-61% respectively.

## 2.4. Sources of Lactic acid bacteria

The niche of the ancestral LAB is first considered soil and plants and next, the gut of herbivorous animals (Morelli et al., 2012). LAB is also associated with animal and plant raw materials and the fermented food products, including dairy, cereal, meat and vegetable, where fermentation can occur. Some LAB also occurs in the infection of the upper respiratory tract like that of common cold and the gastrointestinal and genital tracts of humans and animals (Heilig et al., 2002; Horvath et al., 2009). Isolation of LAB have been reported from a variety of environmental sources like fermented milk products, meat products, fish products, human milk, human and animal feces, droppings of domestic fowls and birds, soil, vegetables, cereals etc. A large number of workers have isolated lactic acid bacteria from different sources and screened them for the use as probiotics (Martinez-Cuesta et al., 1997; Van Neil & Hahn- Hagerdal, 1999). They also reported to play an important role in the production of fermented food (Carvalho et al., 2004). Some representative fermented food products include dairy products like acidophilus milk, acidophilus bifidus milk,

yakult, *kefir*, *koumiss*, acidophilin and *miru- miru* (Yerlikaya, 2014), fermented

sausages (Erkkila & Petaja, 2000), fruits (Fessard et al., 2017), vegetables (Daeschel

& Fleming, 1984; Arroyo-López et al., 2014) and bread products (Corsetti et al.,

2000).

## 2.5. LAB in fermented milk products

Fermented milk products are classified into two different types depending on the type

of microorganisms used. These include the lactic types that include LAB and fungal

lactic type including LAB and yeasts (Mayo et al., 2010). Some naturally occurring

LAB reported from fermented foods are summarised in Table 1.

**Table 1:** Some naturally occurring LAB reported from fermented foods

| Genera/ Species | Product name (Raw material) | Country of origin | References |
|---|---|---|---|
| *Lb. farciminis, Lb. brevis, Lb. alimentarius, Lb. salivarius, Lact. lactis,* | *Chhu* (Yak/ cow milk) | India | Dewan & Tamang  2006 |
| *Enterococcus faecalis, E. faecium, Lactococcus lactis* subsp. *Lactis* | *Datshi* (Cow milk) | Bhutan | Shangpliang et al., 2017 |
| *Lb. farciminis, Lb. paracasei, Lb. biofermentans, Lb. plantarum, Lb. curvatus, Lb. fermentum, Lb. alimentarius, Lb. kefir, Lb. hilgardii, Weissella  confusa, Ent. faecium, Leuc. mesenteroides* | *Chhurpi* (Yak/cow milk) | India | Tamang et al., 2000 |
| *Lb. paracasei, Lb. bifermentans* | *Philu* (Cow/ yak milk) | India | Dewan and Tamang., 2007 |

**Table 1** continued: Some naturally occurring LAB reported from fermented foods

| Genera/ Species | Product name (Raw material) | Country of origin | References |
|---|---|---|---|
| *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *diacetylactis, Lc. lactis* subsp. *cremoris, Strep. thermophilus, Lb. delbruecki* subsp. *bulgaricus* | Shrikhand (Cow, buffalo milk) | India | Sarkar, 2008; Singh & Singh 2014 |
| *Lb. bifermentans, Lb. alimentarius, Lb. paracasei, Lact. lactis, Strep. cremoris, Strep. thermophilus, Lb. bulgaricus, Lb. acidophilus, Lb. helveticus, Lb. cremoris, Ped. pentosaceous, P. acidilactici, W. cibaria, W. paramesenteroides, Lb. fermentum, Lb. delbrueckii* subsp. *indicus,* | *Dahi* (Cow/ Buffalow milk) | India, Bangladesh | Masud et al., 1991; Balasubramanyam & Varadaraj1994 ; Harun-ur-Rashid et al., 2007; Patil et al., 2010 |
| *Lb. brevis, Lb. caucasicus, Strep. thermophilus, Lb. bulgaricus, Lb. plantarum, Lb. casei, Lb. brevis,* | *Kefir* (Goat, sheep, cow) | Taiwan (China), Russia | Chin Wen et al.,1999; Bernardeau et al., 2006 |
| *Lb. helveticus, Lb. kefiranofaciens* | *Airag* (Mare or camel milk) | Mongolia | Watanabe et al., 2008; Yu et al., 2011 |
| *Lc.lactis* subsp. *Lactis, Lc. lactis* subsp. *cremoris, Lactobacillus* sp, *Enterococcus* sp, *Leuconostoc* sp. | *Amasi* (Cow milk) | South Africa | Osvik et al., 2013 |
| *Lb. bulgaricus, Lb. salivarius, Lb. buchneri, Lb. heveticus, Lb. plantarum, Lb. acidophilus* | *Koumiss* (Milk) | Russia, Mongolia | Wu et al., 2009; Hao et al., 2010 |

**Table 1** continued: Some naturally occurring LAB reported from fermented foods

| Genera/ Species | Product name (Raw material) | Country of origin | References |
|---|---|---|---|
| *Lb.fermentum, Lb.plantarum, Lb.helveticus,Leuc.mesenteroides, Ent.faecium,Ent.italicus,Weissella confusa* | *Nunu* (Raw cow milk) | Ghana (West Africa) | Akabanda et al., 2013 |
| *Lb.delbrueckii* subsp. *bulgaricus, Lb. helveticus, Strep. thermophilus* | *Tarag* (Cow/yak/goat milk) | Mongolia | Watanabe et al., 2008 |
| *Lc. lactis subsp. lactis, Lc. lactis subsp. cremoris, Lc. lactis* | *Viili* (Cow milk) | Finland | Kahala et al., 2008 |
| *Leuc. mesenteroides, Ent. faecalis, Strep. lactis supsp. lactis, Strep. cremoris, Lb. casei* subsp. *casei, and Lb. casei subsp. rhamnosus* | *Dadih* (Buffalo milk) | Indonesia | Hosono et al., 1989 |
| *Lc. lactis subsp. cremoris, Lc. lactis subsp. lactis, Lb. delbrueckii subsp. delbrueckii, Lb. delbrueckii subsp. lactis, Lb. helveticus, Lb. casei, Lb. plantarum, Lb. salivarius, Leuconostoc* sp., *Strep. thermophilus, Ent. durans, Ent. faecium* | Cheese (animal milk) | Worldwide | Parente & Cogan 2004; Quigley et al., 2011 |

## 2.6. LAB from GI of healthy human

The beneficial association of LAB with the human gastrointestinal (GI) tract was shown hundred years ago by Moro (1900) and Cahn (1901). The human gut is colonized by millions of microorganisms known as "microbiota" that form diverse structure within the host with various ecological niches that are essential for health (Hooper et al., 2010; Kamada et al., 2013). The GI tract of all humans becomes colonized with bacteria shortly after birth. The pH and retention time of digested food affect the number and species of microorganisms in gut. The low pH of gastric juice causes inhibition of growth of many bacteria. The comparatively neutral pH (usually pH 5-7) and long-lasting maintenance of food in the large intestine with pH 5.7 in descending colon and 6.6-6.8 in rectum of all terrestrial vertebrates are related with an increase in the number and variety of bacterial species (Stevens & Hume 1998). The gut microbiota is first colonised with facultative anaerobes at birth, followed by enterobacteria, coliforms, lactobacilli, and streptococci in the intestine. Colonization by anaerobic genera occurs in rapid succession by *Bifidobacterium, Bacteroides, Clostridium*, and *Eubacterium* (Benno, 1886; Corthésy et al., 2007; Lebeer et al., 2008). Earlier studies on the bacterial fora of the gastrointestinal tract reported 6 to 25% of Bifdobacteria in feces (Finegold et al., 1983) whereas lactobacilli only constituted about 1% in the feces of healthy humans (Hill & Drasar, 1975). When anaerobic culturing techniques are used LAB can rarely exceed $10^8$ CFU per gram (Walter, 2008). Most studies have reported average of around $10^6$ CFU per gram LAB in human fecal sample (Tannock et al., 2000; Walter et al., 2001; Dal Bello et al., 2003).

*L. plantarum* and *L. johnsonii* (Pinto et al., 2006), *Lactobacillus rhamnosus* and *Lactobacillus paracasei* (Verdenelli et al., 2009), *Lactobacillus fermentum* LAB8, *L. mucosae* LAB12, *L. rhamnosus* LAB11, *L. salivarius* LAB9 and *Weissella confusa* LAB10 (Beasley et al., 2006) isolated from feces were selected as candidate probiotic strains based on their quantity in feces, growth density, acid tolerance and antimicrobial activity. In a study, two strains *Weissella confusa* 31 and *Weissella confusa* 20 having probiotic properties were obtained from human feces showed acid tolerance at pH 3, bile tolerance, β-glucosidase activity and adherence to Caco-2 cells (Lee et al., 2012). Similarly, LAB isolated from human infant feces and identified by 16S rRNA sequencing revealed *Lactobacillus (*48%*) and Enterococcus* (38%).

Two strains *L. casei/paracasei* CTC1677 and CTC1678 and *L. rhamnosus* CTC1679 were selected from infant feces were selected for the use as starter cultures in fermented sausages because of its probiotic properties (Rubio et al., 2014).

## 2.7. Role of LAB in cholesterol lowering

Numerous researches on LAB have shown the possession of many health benefits including hypercholesterolemic effect (Table 2). Interest has been focussed on consumption of milk based products and its implication on the plasma cholesterol levels. As elevated level of cholesterol in blood has been regarded as a major factor responsible for heart diseases and safe use of non pharmacological products has been the treatment option to lower lipid levels. Hepner et al. (1979) on feeding pasteurized and non-pasteurized yoghurt observed significant lowering of plasma cholesterol by 5–10% in one week and maintained for 4 weeks. However, on feeding humans the preparation showed no effect. Similarly, no effect in plasma cholesterol was observed

when Pulusani and Rao (1983) fed skim milk, whole milk, 2% fat buttermilk, yogurt,

buttermilk, or sweet acidophilus milk to humans for 3 weeks.

**Table 2**: Probiotic LAB strains claiming hypercholesterolaemic effect

| Genera | Species | Strain | Source | Reference |
|---|---|---|---|---|
| *Lactobacillus* | *acidophilus* | | Probiotic preparation | Lin et al., 1989; Rajkumar et al., 2014 |
| | | | | Lewis et al., 2005 |
| | | L1 | Human | Anderson et al., 1999 |
| | | CHO-220 | | Ooi et al., 2010 |
| | | La5 | Synbiotic product | Asemi et al., 2012 |
| | | 145 | Probiotic yoghurt | Kiessling et al., 2002 |
| | *reuteri* | NCIMB 30242 | Probiotic capsules | Jones et al., 2012 |
| | *helveticus* | KII13 and KHI1 | Fermented Cow Milk | Damodharan et al., 2016 |
| | *casei* | TMC 0409 | Fermented milk | Kawase et al., 2000 |
| | *paracasei* | | Probiotic preparation | Rajkumar et al., 2014 |
| | *paracasei* | LPC37 | Probiotic preparation | Trautvetter et al., 2012 |
| | *bulgaricus* | | Probiotic yoghurt | Asemi et al., 2012 |
| | | | Probiotic preparation | Lin et al., 1989 |

**Table 2 continued**: Probiotic LAB strains claiming hypercholesterolaemic effect

| Genera | Species | Strain | Source | Reference |
|--------|---------|--------|--------|-----------|
| | *plantarum* | A7 (LA7) | Infant feces | Madani et al., 2013 |
| | | EM | *Kimchi* | Choi &Chang 2015 |
| | | HLX37 | Fermented milk | Guan et al., 2017 |
| | *rhamnosus* | GG | | Kim et al., 2016 |
| | *sporogenes* | | Probiotic tablet | Mohan et al., 1990 |
| | *fermentum* | | Probiotic capsules | Simons et al., 2006 |
| *Pediococcus* | *pentosaceus* | KID7 | Fermented finger millet | Damodharan et al., 2015 |
| *Lactococcus* | *lactis* | KF147 | yoghurt | Hassanein et al., 2013 |
| | | | Fermented smooth pigweed leaves | Mariga et al., 2011 |
| *Enterococcus* | *faecium* | | Yoghurt | Agerholm et al., 2000 |
| | | WEFA23 | Infant feces | Zhang et al., 2017 |
| | | M-74 | Probiotic preparation | Hlivak et al., 2005 |

A placebo-controlled design trial was conducted that involved 33 hypercholesterolemia-induced pigs to examine the probiotic containing *L. acidophilus* ATCC 43121 ($2.5 \times 10^{11}$ cells per feeding) for 15 days. The reports showed reduced total blood cholesterol by 11.8% compared to the control (De Rodas et al., 1996).

In a randomized placebo trial study 20 healthy males and 50 females between 18–55 years of age were divided in five different groups, among them three groups were given yoghurt probiotics containing two *S. thermophilus* and two strains of *L. acidophilus* (StLa) which showed no changes in LDL-C and TC (Agerholm et al., 2000a). However, consumption of milk products containing *E. faecium* in a randomized, controlled study among 425 subjects depicted significant decrease in TC by 4% and LDL-C by 5% after 8 week (Agerholm et al., 2000b).

Kiessling et al. (2002) performed randomized, crossover, and placebo-controlled design trial comprising 29 women to evaluate the hypocholesterolemic effect of *L. acidophilus* 145 and *B. longum* 913 in yoghurt. The subjects were administered with 300 g/day yoghurt for 21 weeks' duration and the results obtained showed that HDL-cholesterol was increased significantly ($P < 0.05$) by 0.3 mmol/l and the ratio of LDL/HDL cholesterol was decreased from 3.24 to 2.48. Similarly, another study conducted by Pereira and Gibson (2002) revealed that human isolates *Lactobacillus fermentum* KC5b was able to remove cholesterol by bile salt deconjugation.

A placebo-controlled study was conducted Sindhu and Khetarpaul (2003) to evaluate the effects of a probiotic fermented food on serum cholesterol levels in young Swiss mice. The probiotic cultures used in the study were, *L. casei* NCDC-19 ($10^9$ CFU) and *Saccharomyces boulardii* ($10^9$ CFU). The experimental mice were fed

with a food mixture containing probiotics and 1% cholesterol and the control group was fed food without probiotics, but containing 1% cholesterol for 42 days. After 21 days 19% reduction in the total serum cholesterol was observed, while LDL cholesterol levels were reduced by 37%.

Park et al. (2007) also evaluated the effects of probiotic formulation containing *L. acidophilus* ATCC 43121 ($2 \times 10^6$ CFU/day) on hypocholesterolemic effect in 36 male Sprague-Dawley hypercholesterolemic rats. After 21 days trial the probiotic reduced total serum cholesterol by 25%, and also significantly ($P < 0.05$) reduced very low density lipoprotein (VLDL), intermediate density lipoprotein and LDL cholesterol, compared to the control.

Capsules of synbiotic containing *L. acidophilus* CHO-220 and inulin given to 32 hypercholesterolemic men and women simultaneously reduced TC by 7.84% and LDL-C by 9.27% after 12 weeks (Ooi et al., 2010).

A study on 127 hypercholesterolemic men and women tested with Bile salt hydrolase (BSH) positive *L. reuteri* NCIMB 30242 revealed decrease low density lipoprotein-cholesterol (LDL-C) by 11.64% and total cholesterol (TC) by 9.14% in 9 weeks suggesting the deconjugation of intraluminal bile acids results in reduced absorption of cholesterol and non-cholesterol sterols like plasma campesterol, sitosterol and stigmasterol (Jones et al. 2012a). Similarly on feeding 114 men and women with yoghurt containing microencapsulated strains of BSH-active *L. reuteri* NCIMB 30242 in double-blind, placebo-controlled, randomized trial showed a reduction of 8.92% and 4.81% in LDL-C and TC respectively in 6 weeks, displaying the effectiviness of yoghurt inoculated with BSH-active *L. reuteri* NCIMB 30242 in lowering LDL-C and TC in hypercholesterolaemic subjects (Jones et al., 2012b).

In a recent study, *L. rhamnosus* R4 also demonstrated cholesterol and triglyceride reduction by 50.97% and 28.92%, respectively (Azat et al., 2016).

In a study *Lactobacillus kefir* DH5 isolated from *kefir* significantly lowered the blood cholesterol as well as lumen cholesterol and upreglated peroxisome proliferator-activated receptor - α (PPAR-α) gene in adipose tissues (Kim et al., 2017). This strain showed 100% survivability in *in vitro* gastrointestinal environments and showed a reduction in 51.6% of cholesterol, in addition to this it minimized fatty liver or hepatic steatosis and reduced adipocyte diameter in the high fat diet (HFD) - *L. kefiri* DH5 group indicating a reduction in adiposity by stimulating oxidation of fatty acid.

## 2.8. Mechanisms of cholesterol removal by probiotic lactic acid bacteria

There have been numerous studies on the mechanisms by which probiotics lower cholesterol levels and most of them are from *in vitro* experiments (Gilliland, 1985; Klaver, 1993; Tahri, 1995; Noh, 1997; Usman, 1999; Lin, 2000). These include enzymatic deconjugation of bile acids (Sugano, 1986; Smet et al., 1995; Liong et al., 2006; Lye et al., 2010), cholesterol assimilation by the bacteria (Rasic et al., 1992; Pereira & Gibson 2002) cholesterol binding to the bacterial cell wall (Hosono & Tono-oka 1995; Kimoto et al., 2002) and in some studies conversion of cholesterol to coprostanol have been reported (Lye et al., 2010).

### 2.8.1. Bile acid deconjugation

LAB with active BSH activity have been suggested to lower cholesterol levels through interaction with host metabolism of bile salts (De Smet et al., 1995). Lactobacilli with BSH activity have been reported to have an advantage to survive and colonize the lower small intestine where the enterohepatic cycle takes place, and therefore BSH activity may be considered as an important colonization factor (De
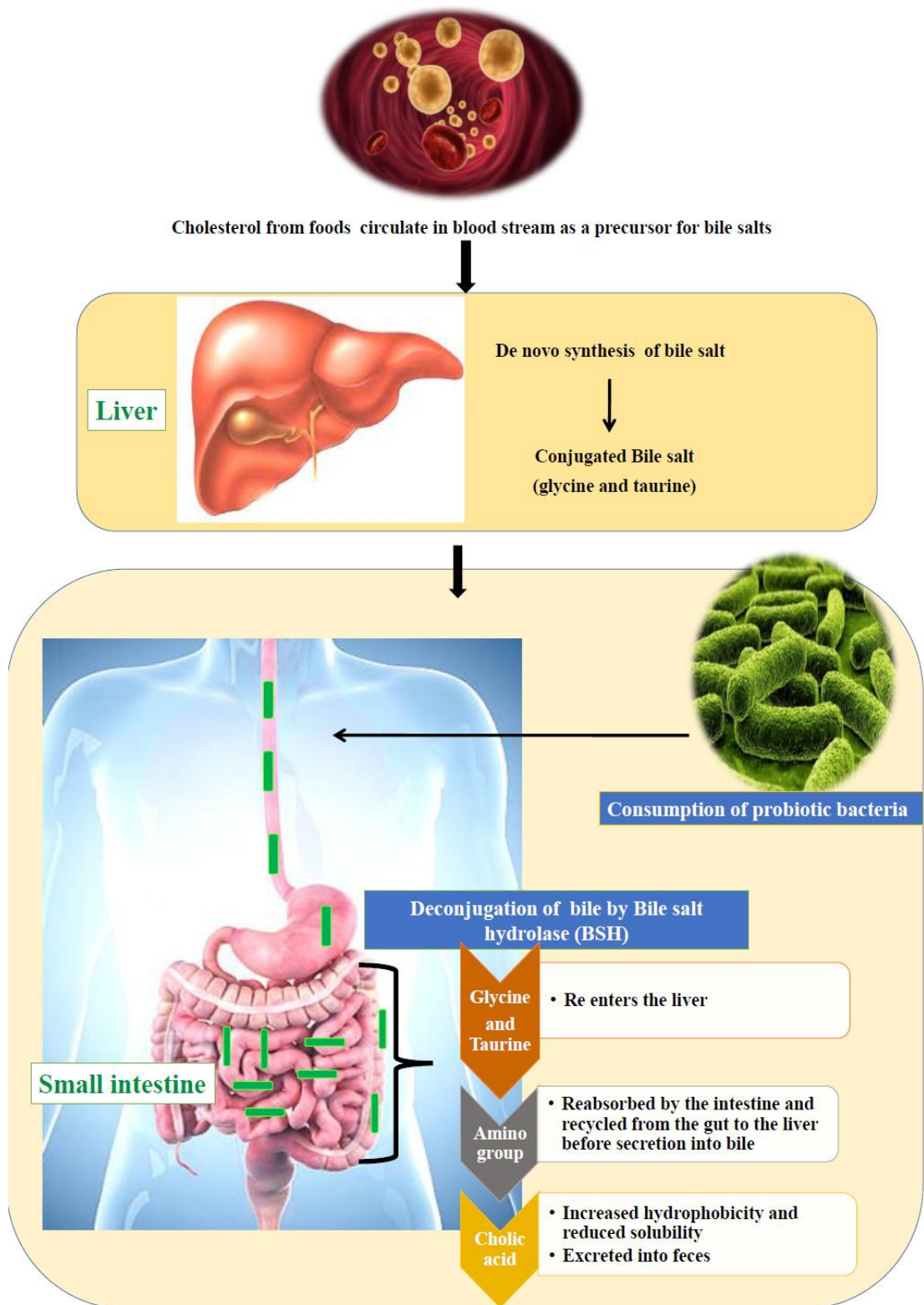
**Figure 2**: Cholesterol as the precursor for new bile acids synthesis and the cholesterol lowering role of bile salt hydrolase (BSH) produced by probiotics

Smet et al. 1995). Some workers (Sander, 1999) proposed the mechanism based on the ability of certain probiotic lactobacilli and bifidobacteria to deconjugate bile acids enzymatically, increasing their rates of excretion (Figure 2). BSH hydrolyze the C-24 N-acyl bond which attaches the bile acid to the amino acid conjugate. BSH enzymes are N-terminal nucleophilic hydrolases (Ntn), similar to penicillin amidases in having a catalytic N-terminal cysteine residue (Tanaka et al., 2000) and are proposed to have evolved as an adaptation to environments containing bile (Begley et al., 2005; Jones et al., 2008).

Bile acid deconjugation by hydrolytic BSH enzyme is one of the most studied mechanisms of cholesterol-reduction (Lye et al., 2010). More than 99% of primary bile acids are conjugated either with the amino acid glycine or taurine after synthesis (Hofmann, 1999). Conjugated bile acid secreted in the small intestine is amphipathic and helps to solubilize hydrophobic vitamins and other dietary fat soluble compounds to be absorbed as mixed micelles (De Aguiar Vallim et al., 2013). Under normal condition conjugated bile salt is efficiently conserved by a process termed enterohepatic recirculation (Carey et al., 1994). After secretion into the intestine at the physiological pH of the intestinal lumen, the vast majority bile salts (conjugated and deconjugated) can be re-absorbed, either through active transport in the terminal ileum and possibly the jejunum for conjugated bile salts, or passive diffusion in the small and large intestine for unconjugated bile salts (Trauner & Boyer, 2003). Conjugated bile salts are transported by active transport while unconjugated bile salts are transported by passive transport. The reabsorbed bile acids entering the bloodstream and are taken up by hepatocytes in the liver, reconjugated, and re-secreted into bile. About 5% of the total bile acid pool (0.3 to 0.6 g) per day escape epithelial absorption and may be extensively modified by the indigenous intestinal

bacteria (Bortolini et al., 1997).The free bile acid (deconjugated bile salt) is more hydrophobic than conjugated bile salt, thereby resulting in lower absorption in the intestinal lumen and lost in feces. Thus, in a stable condition, deconjugation of bile salts can reduce serum cholesterol levels by increasing the formation of new bile salts that are needed to replace those that have escaped the enterohepatic circulation (Reynier et al., 1981).

Some strains of *Lactobacillus acidophilus* were found to secrete BSH which catalyses the hydrolysis of glycine and/or taurine conjugated bile salts into amino acid residues and free bile salts (bile acids) (Corzo & Gilliland, 1999). Unconjugated bile acids are more hydrophobic than the conjugated biles. The increase in concentrations of bile acids have been reported to solubilise membranes and cause the integral membrane proteins dissociation (Coleman et al., 1980 Heuman et al., 1996), causing in leakage of intracellular contents (Noh et al., 1993 and Fujisawa et al., 1996). These free bile salts being less soluble cannot be re-absorbed and can be further transformed into secondary bile acids the accumulation of which in the colon has been contemplated to cause certain tissue damage (Li & Chiang, 2012).  In some bacterial strains BSH may function as a medium to acquire a source of energy and building blocks for biosynthesis, through the deconjugation of bile salt to free glycine and taurine (Camilleri & Gores, 2015; Ridlon et al., 2016) . In the small intestine the increased BSH enzyme activity disrupts micelle formation and absorption of cholesterol and lipids (Sugano, 1986; Liong et al., 2005; Ridlon et al., 2016).

Bile salts found in feces are of mostly deconjugated forms consisting of entirely of deoxycholate and lithocholate (Korpela et al., 1988).  It was observed in *in-vivo* human trials that the strains of *L. acidophilus* could deconjugate bile salt *in-vitro* produced higher faecal excretion of deoxycholate because of deconjugation of

glycine or taurine by the transformation of cholate into deoxycholate through 7α-dehydroxylation of other intestinal bacteria (Ahn et al., 2003).

The pH of upper intestinal tract is 5.50-6.50 where half of the free bile salts and few glycine-conjugated bile salts were found to be protonated (nonionized), while no protonation occurred in taurine-conjugated bile salt (Carey & Cahalane, 1988). It was observed that significantly higher rates of deconjugation of glycodeoxycholate (GDCA) than taurodeoxycholic acid (TDCA) by *Lactobacillus plantarum*. While the BSH negative isolates on comparison with the wild type exhibited pH and concentration dependent toxicity of GDCA and not with TDCA (De Smet et al., 1995). It was reported that BSH active bacteria may be able to use the amino acid, taurine, as an electron acceptor which can improve growth (Van Eldere et al., 1988). Corzo and Gilliland (1999) reported in an *in vitro* experiment resembling human intestinal pH of 6.5 and glycocholate to taurocholate ratio of 2:3, the glycine conjugated bile salt was found to be more efficiently deconjugated by strains of *L. acidophilus* than taurine conjugated bile salt showing solubility of both conjugated bile salts under acidic conditions (Corzo & Gilliland, 1999). This can be explained by the pKa values of taurine and glycine conjugated bile salts, and of deconjugated bile salts, which are at pH 1.9, 3.9 and 5.0 respectively. In such condition deconjugated bile salts are protonated and precipitated, while taurine-conjugated bile salts remain ionized in solution, and glycine-conjugated bile salts are partially precipitated without hydrolysis (Dashkevics & Feighner, 1989). Klaver and van der Meer (1993) reported that the degree of deconjugation by *L. acidophilus* RP32 was higher under more acidic conditions than at pH 6.0. This study confirmed that the removal of cholesterol was due to its co precipitation with deconjugated bile salts because of an acidic environment. Brashears et al. (1998) hypothesized that cholesterol removal was not

only as a result of bile salt deconjugation, co-precipitation of cholesterol with deconjugated bile salt or destabilization of cholesterol micelles. However, low pH and strong deconjugation activities were reported to be important factors for the precipitation of cholesterol *in-vitro* (Ahn et al., 2003). The regulation of BSH activity by pH is still unclear although BSH activities were shown to be higher in lower pH values. Various strains of the same bacterial species also exhibited different BSH activity under similar pH levels (Lunden & Salvage, 1990; Corzo & Gilliland, 1999). Tannock et al. (1989) disapproved the hypothesis of deconjugation because free bile acids are more cytotoxic than their conjugates. It was suggested that deconjugation may be a detoxification mechanism which is of vital importance to the *Lactobacillus* cell (De Smet et al., 1995). It has also been suggested that BSH act as a detergent shock protein that protects the producing bacteria from the bile acids toxicity in the gastrointestinal tract (Adamowicz et al., 1991; Flahaut et al., 1996).

The molecular fate of the amino acid released by the deconjugation is unclear. However, study by Tanaka et al. (2000) revealed that BSH isolated and purified from *B. longum* SBT 2928 was an intracellular enzyme and hydrolysis of bile salts makes released amino acids available for cells.

Many BSH active probiotic strain have been reported which include, *Lactobacillus acidophilus* CHO-220 (Wood & Holzapfel, 1995), *Lactobacillus plantarum* (De Smet et al., 1995; Boever & Verstraete, 1999), *Lactobacillus acidophilus* NBHK008 (Corzo & Gilliland, 1999), *Lactobacillus johnsonii* 100-100 (Wood & Holzapfel, 1995; Elkins et al., 2011), *Lactobacillus casei* (Wood & Holzapfel, 1995; Brashears et al., 1998; Corzo & Gilliland, 1999), *Lactobacillus rhamnosus* NBHK007 (Tanaka et al., 1999; Moser & Salvage, 2001), *Lactobacillus acidophilus* 4356 and 4962 (Liong & Shah, 2004), *Enterococcus faecium* FAIR-E 345

(Wijaya et al., 2004), *Lactobacillus reuteri* NCIMB 30242 (Branton et al., 2011), *L. paracasei* subsp. *paracasei* (Moser & Salvage, 2001), *Pediococcus acidilactici* NBHK002 (Tsai et al., 2014), *Lactobacillus acidophilus* ATCC 33200 (Tsai et al., 2014), etc.

However, it was proposed that cholic acid (free bile acid) was produced by BSH activity in the intestine, could accumulate inside the bacterial cells when the bacteria were active (Kurdi et al., 2003). This entrapment of free bile acids by bacterial cells could contribute to the decreased production of secondary bile acids, which are considered cytotoxic and precarcinogenic (Kurdi et al., 2003).

BSH genes may be either located in the chromosome or in plasmid, like the megaplasmid identified in *L. salivarius* UCC118 (Claesson et al., 2006). Several strains of *Lactobacillus plantarum* WCFS1 contain more than one BSH homolog, which is not identical (Begley et al., 2006). Sequencing of the homologs revealed that the genetic arrangement of *bsh* regions was not the same in all strains and there were cases where more than one homolog was present. Such nultiple homologs were not located in the same portion of the chromosome (Begley et al., 2006). It had earlier been hypothesized that BSH genes may be acquired horizontally among intestinal microorganisms (Begley et al., 2006), however, there is no forceful evidence predicting horizontal transfer of BSH genes in intestinal microorganisms (Geng & Lin, 2017). Many studies reported that the BSH subunit has a molecular weight in the range from 28 to 50 kDa, and have an optimal pH 3.5 to 6 for BSH activity. The identified BSH enzymes are reported to display activity up to 60 °C temperature (Taranto et al., 1995; Corzo & Gilliland, 1999; Geng & Lin, 2017). Whole genome sequencing of *L. acidophilus* NCFM revealed that this strain possesses two *bsh* genes (*bshA* and *bshB*). These sequences of the BSH enzymes encoded by specific position

in the gene share a higher degree of similarity to BSH enzymes from other *Lactobacillus* species than to each other, indicating that they may have been acquired from different sources (McAuliffe, 2005). Works on the identification, characterization and crystallization of BSH enzymes from various bacterial species other than lactic acid bacteria have been conducted. The *bsh* genes of LAB such as *Lactobacillus plantarum* (Christiaens et al., 1992), *Lactobacillus johnsonii* (Elkins & Savage, 1998), *Lactobacillus gasseri* (Russel et al., 2001), *Lactobacillus acidophilus* (GenBank accession no. AF091248.3) and Enterococci (Wijaya et al., 2004) have been cloned and characterized. *L. salivarius* (Xu et al., 2016), and *E. faecalis* (Kumar et al., 2013) were identified and cloned a gene encoding the BSH enzyme from *L. fermentum* NCDO394 (Rani et al., 2017). In another study, a BSH gene from the genome of *L. salivarius* NRRL B-30514 was identified from chicken (Wang et al., 2012). Similarly, a putative BSH gene (981 bp) from the genome of *L. gasseri* FR4 encoding 326 amino acids was identified by Rani et al. (2017). The LgBSH (BSH gene of *L. gasseri* FR4) was found to be 37 kDa on SDS–PAGE that was similar to the molecular mass of the BSH enzyme earlier reported as 37 kDa on SDS–PAGE by Wang et al. (2012), Kumar et al. (2013) and Jayashree et al. (2014). Most identified BSH enzymes have a narrow substrate spectrum and show a much higher activity in hydrolyzing glycine conjugated bile salts than taurine-conjugated bile salts (Tanaka et al., 2000; Kim et al., 2004; Liong & Shah, 2005; Pavlovic et al., 2012). The functions of BSH enzyme include; 1) they play a nutritional role by deconjugating the amino acids liberated from bile salt that could potentially be used as carbon, nitrogen, and energy source, 2) they facilitate in the incorporation of cholesterol and bile salt in the membrane thereby increasing the tensile strength of the membranes (Boggs et al., 1987) or change fluidity, 3) they cause bile detoxification and 4) BSHs may combat

the harmful effects of bile increasing the persistence of a bacterium in the gastrointestinal region (Begley et al., 2006; Kumar et al., 2012).

## 2.8.2. Cholesterol incorporation in cellular membrane

Noh et al. (1997) suggested that *L. acidophilus* incorporates some of the cholesterol removed from the medium into their cellular membrane during growth. In the study the cells of *L. acidophilus* ATCC 43121 were grown in the presence of oxgall to form cholesterol micelles, the micelles showed greater resistance to lysis by sonication than did the cells grown in control broth. This result suggested that the cholesterol incorporation into the cellular membrane or wall of the lactobacilli may have altered them thereby making them more resistant to sonic disruption. Attached or incorporated cholesterol into or to the bacterial cells would more likely be less available for absorption from the intestine into the blood (Liong & Shah, 2006). This mechanism involves a link between incorporation of cholesterol and pH of the growth medium. The bacterial cultures grown without pH control were found to significantly contain more cholesterol compared with cultures grown under controlled pH of 6.0. However, the amount of cholesterol removed from the membrane portion of *L. acidophilus* was not equal to the total amounts removed by the culture. Cholesterol incorporation had been earlier reported in mycoplasma by Razin (1980) and Melchior and Rottem (1981). It was suggested that the cholesterol in the cell membrane provides tensile strength and protects from lysis (Razin, 1967). The cholesterol binding ability of bacteria was compared to that of mycoplasma and also whether cell wall interferes or participates in binding. *L. acidophilus* ATCC 43121 grown in presence of ox gall and cholesterol micelles showed greater resistance to lysis by sonication than in control tube, suggest that cholesterol may have altered the cellular membrane or wall making them resistance to lysis. The cells of *L. acidophilus* that

were grown in the presence of oxgall and cholesterol micelles did not give a Gram positive reaction while those without cholesterol micelles did. These results suggested that changes in the bacterial cell had occurred in presence of cholesterol and bile salts. The study on the effect of Tween 80 on cholesterol uptake suggested the increase in assimilation upto 0.05% followed by a decrease in assimilation beyond this concentration. Similarly the effect of Tween 80 among strains of *L. bulgaricus* survived freezing and was suggested to be related to alterations in the cell membrane (Smittle et al., 1974) suggesting that changes in the bacterial cell had occurred in presence of cholesterol and bile salts.

Cholesterol removal was studied in strains of lactococci grown media (Kimoto et al., 2002) revealed that the presence of cholesterol had altered the fatty acid composition of the cells. The concentration of saturated and unsaturated fatty acids in the lactococcal cell membrane had raised causing increase in the membrane strength and subsequently being resistant to lysis (Kimoto et al., 2002). In another study, the probable occurrence of cholesterol incorporation within the lipid bilayer was investigated (Lye et al., 2010). He designed fluorescence probes and incorporated them into the membrane bilayer of probiotic cells and grew them in absence and presence of cholesterol. He observed addition of cholesterol in the regions of the phospholipid tails, upper phospholipids, and polar heads of the cellular membrane bilayer in cells grown in the presence of cholesterol compared to the control cells, indicating incorporation of cholesterol in those regions.

### 2.8.3. Cholesterol assimilation

Dambekodi and Gilliland (1998) proposed another hypothesis stating cholesterol removal may be contributed by assimilation of cholesterol, and not bile salt

deconjugation. Three *Lb. acidophilus* strains were able to assimilate cholesterol in MRS broth during 18 h culture in 37 °C (Rasic et al., 1992) with the assimilation value being higher than that of other species and strains of LAB studied. In another study, two strains, *L. acidophilus* strains ATCC 43121 and NCFM-L in frozen and refrigerated condition were studied for cholesterol assimilation (Piston & Gilliland, 1994). During the 28 days frozen storage period there was no significant difference in bile tolerance and cholesterol assimilation. However, decrease in viability of one strain and cholesterol assimilation was observed under refrigerated storage conditions of non-fermented milk, whereas the other exhibited greater viability and subsequently better cholesterol assimilation abilities. It was hypothesized that the decrease in activity during refrigerated storage also caused a decrease in activity *in-vivo* and suggest cholesterol assimilation to be growth dependent. However, this supported a study which reported the cholesterol assimilation ability in bacteria being highly dependent on their growth and the growth stage at which the inoculums are used (Pereira & Gibson, 2002). Although there may be several factors affecting growth such as pH, bile and origin of probiotics on the nature of cholesterol assimilation. *In-vitro* study on cholesterol assimilation abilities by Pereira and Gibson (2002) among nine strains of lactic acid bacteria and bifidobacteria (human origin) along with commercial strains were investigated. The study suggested that strains did not grow well in medium containing bile salts and were unable to assimilate cholesterol to the extent as those in the presence (Pereira & Gibson, 2002).

This was in agreement with various studies that showed strains of bacteria having higher ability to remove cholesterol from culture medium in presence of higher concentrations of bile salts (Gilliland et al., 1985; Rasic et al., 1992). More cholesterol assimilation was seen in the medium containing 0.006 M taurocholate than

from those containing any other level (Dambekodi & Gilliland, 1998). However on the other hand it was suggested by Walker and Gilliland (1993) that there was no significant correlation between the ability of *L. acidophilus* to deconjugate bile acids and the ability to assimilate cholesterol.

The conversion of cholesterol to coprostanol is studied as it decreases the amount of cholesterol being absorbed, progressing to a reduced concentration in the total cholesterol pool (Kumar et al., 2012). The microbial conversion of cholesterol to coprostanol is a rare event, and only some few strains had been studied (Sadzikowski et al., 1977; Freier et al., 1994). Earlier it was reported that *Eubacterium* in the gut coverts cholesterol to coprostanol as its end product via an indirect pathway (Ren et al., 1996). As coprostanol is poorly absorbed and excreted in feces, early studies tried to isolate those gut bacteria to lower blood cholesterol (Illman et al., 1993; Freier et al., 1994). In a study, it was observed that cholesterol dehydrogenase or isomerase produced by *Sterolibacterium denitrificans* was responsible for causing the transformation of cholesterol to cholest-4-en-3-one, an intermediate cofactor in the conversion of holesterol to coprostanol (Chiang et al., 2008). Lye et al. (2010) studied the conversion of cholesterol to coprostanol in three probiotic strains *Lactobacillus acidophilus, L. bulgaricus,* and *L. casei* ATCC 393. He reported the presence of both intracellular and extracellular cholesterol reductase signifying possible conversion of cholesterol to coprostanol that decreased the concentration of cholesterol in the medium in fermentation condition.

### 2.8.4. Binding of cholesterol to the cells

Another mechanism of cholesterol removal is adhesion of the cholesterol to the cell surface. LAB including lactococci, bind cholesterol to the cells was reported by Hosono and Tono-oka (1995). Cholesterol solution in 60% ethanol was mixed with

the lyophilized cells to study the cholesterol-binding ability, where they suggested that cholesterol binding may be a physical phenomenon related to the cell wall. However, the growth of cells in 60% ethanol was not considered.

In a study the whole cell of *B. Longum* membrane fraction showed less cholesterol per milligram of protein indicating cholesterol was not closely associated with the membrane, but merely attached to the cell surface (Dambekodi & Gilliland, 1998). A study conducted by Tahri et al (1995), indicated more than 40 percent of the cholesterol was extracted by sonication from cells of *Bifidobacterium breve* ATCC 15700, however the absorbed cholesterol could not be obtained even after several washings thus proving the powerful binding between cholesterol and growing cells.

A study was conducted to study the mechanism of cholesterol removal by seven different strains of Lactococci (Kimoto et al., 2002). It was observed that *Lc. lactis* subsp. *lactis* biovar *diacetylactis* N7 removed cholesterol from media during growth. Similarly, the heat-killed cells of strain N7 were also tested for the mechanism and it was observed that these cells could not take up cholesterol, but could remove it from media as it seemed that some cholesterol had bound to the cells. There was also difference in the cholesterol level among the live growing cells and the heat-killed cells of strain N7. The cholesterol removal by the actively growing cells of strain N7 was higher. This experiment leads to the hypothesis that cholesterol removal was not only successful by the living cells but also by binding to the dead cells.

# Methods and Material

## 3. Methods and materials

### 3.1. Chemicals and reagents

All the chemicals used were of analytical grade obtained from Hi Media, India; Merck, India; Sigma, States United. The cholesterol testing kit was obtained from ERBA diagnostics Mannheim, Germany. The microorganisms used as indicator or control strains were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The nucleic acid primers were obtained from Imperial Life Sciences, Chandigarh, India.

### Composition of culture media

1. *Lactobacillus* MRS broth*

| Composition | $gL^{-1}$ |
|---|---|
| Peptone | 10.0 |
| Beef extract | 10.0 |
| Yeast extract | 5.0 |
| Glucose | 20.0 |
| Sodium Acetate | 5.0 |
| Tri sodium citrate | 2.0 |
| $Na_2HPO_4$ | 2.0 |
| Tween 80 | 1.0 |
| $MgSO_4.7H_2O$ | 0.1 |
| $MnSO_4.5H_2O$ | 0.05 |

**Preparation of medium:** The components were added to distilled water, mixed thoroughly. The media was sterilized by autoclaving. The pH was maintained at 6.5±0.2 at 25 °C. * For MRS Agar add 2% of agar in above composition. For isolation 1.5% $CaCO_3$ was added.

2. **Medium for screening cholesterol lowering**

| Composition | gL$^{-1}$ |
|---|---|
| **MRS broth, cholesterol and ox gall broth** | |
| Peptone | 10.0 |
| Beef extract | 10.0 |
| Yeast extract | 5.0 |
| Glucose | 20.0 |
| Sodium Acetate | 5.0 |
| Tri sodium citrate | 2.0 |
| $Na_2HPO_4$ | 2.0 |
| Tween 80 | 1.0 |
| $MgSO_4.7H_2O$ | 0.1 |
| $MnSO_4.5H_2O$ | 0.05 |
| Oxgall | 30 |
| Polyoxyethanyl-cholesteryl sebacate | 70-100 µg/ml |

**Preparation of medium:** The components were added to distilled water, mixed thoroughly. The pH was maintained at 6.5±0.2 at 25 °C. The media was sterilized by

autoclaving. Polyoxyethanyl-cholesteryl sebacate (Sigma, United States was added to a concentration of 70-100 µg/ml after filter sterilization.

| 3. Medium for gas production from glucose | |
| --- | --- |
| Composition | gL$^{-1}$ |
| Peptone | 10.0 |
| Beef extract | 10.0 |
| Yeast extract | 5.0 |
| Dextrose | 5.0 |
| Sodium Acetate | 5.0 |
| KH$_2$PO$_4$ | 1.0 |
| Tween 80 | 1.0 |
| MgSO$_4$.7H$_2$O | 0.2 |
| MnSO$_4$.5H$_2$O | 0.05 |

**Preparation of medium**: The components were added to distilled water, mixed thoroughly, 5ml of broth medium was dispensed in test tubes and Durham tube was placed in inverted position without air bubble. pH 7.1±0.2 was maintained and media was sterilized by autoclaving.

**4. Muller Hinton Agar**

| Composition | gL$^{-1}$ |
|---|---|
| Beef extract | 2.0 |
| Casein hydrolysate | 17.50 |
| Starch | 15.0 |
| Agar | 2.0 |

**Preparation of medium:** The components were added to distilled water, mixed thoroughly. pH 7 at 25 $^{o}$C was maintained and media was sterilized by autoclaving.

**1.  Nutrient broth\***

| Composition | gL$^{-1}$ |
|---|---|
| Peptone | 5.0 |
| Beef extract | 3.0 |
| NaCl | 5.0 |

**Preparation of medium:** The components were added to distilled water, mixed thoroughly. Media was sterilized by autoclaving.

\* For Nutrient Agar add 2% of agar in above composition.

**Reagents and chemicals**

1.     Agarose (HiMedia,  India)

2. Anaerobic gas Pack system (LE002) (HiMedia, India)

3. Cholesterol testing kit ERBA (Mannheim, Germany )

4. Cholic acid (HiMedia, India)

5. Chloroform (HiMedia, India)

6. DNA isolation kit (HiMedia, India)

7. Egg yolk lecithin (Sigma, Unijted States)

8. Ethidium bromide (HiMedia, India)

9. Ethyl acetate (HiMedia, India)

10. Ezy MIC™ Strips (HiMedia, India)

11. Furfuraldehyde (HiMedia, India)

12. Glacial acetic acid (HiMedia, India)

13. Glyine (HiMedia, India)

14. GoTaq® Green Master Mix 2 X (Promega, USA)

15. Hexadecane (HiMedia, India)

16. Hi carbo ™ kit (HiMedia, India)

17. Ox gall (HiMedia, India)

18. Primer 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3')

19. Primer Leu F (5'-GTGCCTAATACATGCAAGTCG-3') and LeuR (5'-AGCTTCAAAGGTAGTCAAGAC -3')

20. Polyoxyethnayl cholesterol sebacate (Sigma, Unijted States)

21. Ringer Solution (Merck, Germany),

22. Sodium thioglycolate (HiMedia, India)

23. Sodium glycholate (Sigma, Unijted States)

24. Sodium taurocholate (Sigma, Unijted States)

25.    Sucrose (HiMedia, India)

26.    Taurodeoxycholic acid (TDCA) (Sigma, Unijted States)

27.    Taurine (HiMedia, India)

28.    Trichloroacetic acid (HiMedia, India)

29.    Tween 80 (HiMedia, India)


**Preparation of reagents and chemicals**

1. **0.85% saline:** 0.85 g of NaCl was dissolved in 90 ml of distilled water and the volume was made up to 100 ml. It was sterilized by autoclaving.

2. **Gram's crystal violet:** Crystal violet (20 g) was dissolved in little amount of water followed by addition of 8.0 g of ammonium oxalate and the volume was made up to 800 ml. Further 200 ml of ethanol was added and stored in amber color bottle for 48 h. It was filtered before use.

3. **Gram's Iodine:** Iodine (3.3 g) and potassium iodide (6.6 g) was dissolved in 100 ml of $dH_2O$ and the volume was made up to 1l. It was stored in amber color bottle and kept for maturation at room temperature for 48 h.

4. **Counter stain Safranin:** Safranin (2.5 g) was dissolved in 100 ml of $dH_2O$ and the volume was made up to 900 ml. To this 100ml of ethanol was added. It was stored in amber color bottle and kept for maturation at room temperature for 48 h.

5. **1 N hydrochloric acid:** A volume of 0.6 ml of concentrated HCl was added to 7.5 ml of $dH_2O$ and mixed well. The volume of the solution mixture was made up to 50 ml with $dH_2O$.

6. **1 N Sodium hydroxide**: An amount of 4.0 g of Sodium hydroxide was dissolved in 100 ml of $dH_2O$.

7. **Potassium hydroxide (3%)**: The potassium hydroxide (3 g) was dissolved in 20 ml of $dH_2O$ and the volume was made up to 100 ml with $dH_2O$.

8. **Hydrogen peroxide solution (10%)**: One millilitre of 10% hydrogen peroxide solution was diluted to 100 ml with $dH_2O$ and was kept in brown bottle. This was prepared freshly prior to use.

9. **Tris (1 mol $L^{-1}$, pH 8)**: An amount of 12.11 g of tris was dissolved in 80 ml of $dH_2O$ and pH was adjusted to 8 followed by making of volume up to 100 ml with $dH_2O$. It was sterilized by autoclaving.

10. **TAE Electrophoresis buffer preparation:** A 50X stock solution was prepared by dissolving 242 g Tris base in water in 57.1 ml glacial acetic acid, and 100 ml of 500 mM EDTA (pH 8.0) solution, and bringing the final volume up to 1 liter.

**Reference strains:**

**List of microorganisms used as reference strains**

| S.No | MTCC No. | Microorganism Name | Purpose of use |
|------|----------|--------------------|-----------------|
| 3. | 1089 | *Escherichia coli* | Antibiotic susceptibility |
| 4. | 7443 | *Staphyloccus aureus* | Antibiotic susceptibility |

### 3.2. Methodology of Survey

The data was collected by the method of open question and face to face interviews (Annexure I -Questionnaire).The information on traditional management practices of yak and the technology of processing yak milk into fermented milk were obtained from the respondents with the help of a set of questionnaire. The area of study was East district of Sikkim (Tsmogu lake region and 9[th]Mile) and North District of Sikkim

(Lachen and Lachung), since maximum yak populations are found in these regions. Random samples of the fermented milk products were collected from of 35 households were collected. The valuable information from the herder community on the use of traditional practices in yak rearing and the maintenance of their livelihood inquired. The ethnic processes on how the yak milk was molded into valuable fermented milk products were understood along with milk processing, methods of preparing fermented meat items. Whether any value added products made from yak by tradidional practices were also inquired.

Previously isolated fecal LAB from the Laboratory of Dept. of Microbiology, Sikkim University was used. These LAB isolates were collected from healthy individuals considering those who had not taken antibiotics for the last one month in East Sikkim.

### 3.3. Collection of Samples

Samples of fermented milk products of yak namely *Shyow* (Dahi in Nepali*), Marr* (Ghew in Nepali), *Thara* (Mohi in Nepali), *Chhurpi* (Hard and Soft type*)* were collected from Eastern and Northern part of Sikkim (number of samples (n) = 4 each type). Samples were collected in sterilized containers. All the samples were transported to the laboratory in ice pack and preserved at $-20\ ^oC$.

### 3.4. Isolation of Lactic acid Bacteria (LAB) from fermented Yak milk products

Each sample of *Shyow* (Dahi in Nepali*), Marr* (Ghew in Nepali), *Thara* (Mohi in Nepali), Hard and Soft *Chhurpi* was suspended at an amount of 10 gm of sample in 100 ml physiological saline, 0.85% NaCl solution (in a ratio of 1:10) in an Erlenmeyer flask. The flask was thoroughly shaken. The liquid samples were directly suspended in saline water but the solid ones were first soaked in sterile distilled water

and grinded into paste before suspending. Then 1 ml of the homogenized suspension was taken and added to 9 ml physiological saline in a test tube, vortexed for 1 min and dilutions were made serially upto $10^{-8}$.

De Man, Rogosa and Sharpe (MRS) agar (De Man et al., 1960) media supplemented with 1% of $CaCO_3$ was used for plating. 1ml of each dilution was added to each stelilized petri plate and the medium was poured. The plates were incubated at 30 $^{o}$C in anaerobic gas pack system (LE002) for 72 h. The bacterial colonies encircled with a clear zone or halos showing acid production were selected (Leisner et al., 1997). The pure culture of the isolates was obtained by streaking on MRS Agar without $CaCO_3$. Each bacterial isolates was preserved in glycerol stock containing using 50% (v/v) glycerol at −80 $^{o}$C. All working cultures were preserved in MRS agar slants at 4-8 $^{o}$C.

## 3.5. Preliminary screening

**3.5.1. Colony morphology:** Colony morphology of the bacterium was studied from their growth on surface of MRS agar medium.

**3.5.2. Shape and Character:** To determine the shape and Gram staining character, a bacterial smear was made from overnight grown broth culture in a grease free slide and stained by the Grams staining method (Ni et al., 2015). It was done by picking the colonies with a sterile loop, culture was smeared on a clean glass slide and heat fixed followed by flooding the slide with crystal violet for 30 sec. The slides were rinsed with running water for 5 sec and flooded with the Gram's iodine for 1min. Again the slides were rinsed with water followed by decolourisation of slides using 95% ethanol for 20 to 30 sec. Then the slides were rinsed with water again and stained with a counter stain safranin for 60 sec and excess stain was removed by

rinsing with water. After drying, the slides were examined under oil immersion in the compound microscope.

**3.5.3. Catalase test:** The cultures were grown in MRS agar broth for 24 h and then the production of gas bubbles were observed by adding 0.5 ml of 10 % hydrogen peroxide solution (Merck) to the drop of overnight grown cultures. Effervescence produced indicates the presence of catalase (Schillinger & Lucke, 1987).

**3.5.6. Potassium Hydroxide (KOH) test:** This test is also known as string test. To a clean grease free slide 3% KOH solution was added and a loop full of fresh culture was mixed in a 1 to 2 cm area on the glass slide for a maximum of 1min and the loop was slowly lifted, observe for the formation of a string. The absence of string formation was observed in Gram positive bacteria (Gregersen, 1978).

## 3.6. Screening of LAB isolates for cholesterol lowering

The LAB isolates were screened for cholesterol removal or lowering potential by the method described by Liong and Shah (2005). Freshly prepared MRS broth was supplemented with 0.30% oxgall as a bile salt. Polyoxyethanyl-cholesteryl sebacate (Sigma, United States), water soluble cholesterol was filter sterilized. The filter sterilized cholesterol was added to the sterilized MRS broth to make the final concentration of 70-100 $\mu$g/ml and 5 ml total volume. The broth was then inoculated with each overnight grown fresh isolate at 1%. The capped tubes were then incubated anaerobically at 30 $^{o}$C for 20 h.  The cholesterol concentration in the MRS broth was assayed the contents of the tubes were centrifuged in centrifuge machine (Micro CL 21R, Thermo Scientific, United States) at 6000 rpm for 7 min at 4 $^{o}$C (Ziarno et al., 2007). The remaining cholesterol in the broth was determined using *in vitro* diagnostic testing kit (Erba Manneheim, Mannheim, Germany) with slight modifications.  Absorbance was read at 505 nm in UV/VIS spectrophotometer

(Lambda 25, Perkin Elmer, Wokingham, United Kingdom) after incubation at 37 $^{o}$C for 10 min. All experiments were replicated twice. The concentration of cholesterol in the spent broth was determined using a standard curve of Polyoxyethanyl-cholesteryl sebacate (Sigma, United States) and the cholesterol lowering percentage by the formula given below.

$$Cholesterol\ lowering\ \% = [(C1 - C2) \div C1] \times 100$$

Where, C1 is cholesterol concentration of the uninoculated control tube and C2 is cholesterol concentration of the test.

## 3.7. Biochemical characterization to the genus level

The isolates screened were further characterized to the genus level following Axelsson (2004). The isolates were grouped according to eight characters namely tetrad formation, $CO_2$ production from glucose, growth at two different temperatures, growth at two different salt concentrations and growth at two different pH.

### 3.7.1. Gas (CO2) production from glucose

MRS broth tubes of 10 ml, without citrate and containing inverted durham tubes was inoculated with 24 h old cultures and incubated at 30 ºC (Schillinger & Lucke, 1987). Accumulation of gas in the inverted durham tubes indicated production of $CO_2$ production from glucose.

### 3.7.2. 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 filter sterilized, cooled to room temperature and inoculated with 24 h old MRS broth

culture. The tubes were incubated at 30 ºC for 24-72 h and observed for growth (Dykes et al., 1994).

### 3.7.3. Growth at different temperatures

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

### 3.7.4. Growth in different salt concentrations

The isolates were grown in varied level of NaCl concentrations. Tolerance to salt was tested by inoculating a loop-full of culture in tube containing MRS broth supplemented with 6.5 %, 10.0 % and 18.0 % NaCl, respectively, and incubated for 3 days at 30 ºC in a slanting position to improve aeration (Schillinger & Lucke, 1987). Cultures were observed for growth after incubation for 72 h.

### 3.8. Screening for probiotic characteristics

The LAB isolates showing cholesterol reduction lowering were selected for studied for the following probiotic characteristics.

### 3.8.1. Tolerence to low pH

The ability to resists acidic pH of pH 2.5 and 2 by the bacterial isolates were studied. Sterile MRS broth supplemented with 0.30% Oxgall (HiMedia, India) was adjusted to a pH of 2.5 with HCl. (Liong & Shah, 2005). Before inoculation the cell density of the overnight grown fresh cultures isolates was maintained to an OD value of 1 at 600 nm. Then 1% (v/v) of each test culture was added to the broth and incubated at 30 °C. 1 ml bacterial samples were withdrawn every 30 min upto 2 h and serial dilutions were made in peptone water. The tubes were vortexed and 1 ml each was plated in

MRS agar and the plates were incubated at 30 °C for 24 h in an anaerobic condition. After incubation the colony forming unit was calculated in each batch and tolerance was determined by comparing the final count after 2 h with the initial count at 0 h. The experiments were repeated twice. Similarly, the tolerance was determined at pH 2.0 also.

### 3.8.2. Tolerance to Bile salt

The method used for determining bile tolerance was similar to that described by Gilliland et al. (1984). However, three different types of bile salts namely oxgall (HiMedia, India), cholic acid (HiMedia, India) and taurocholic acid (TDCA) (Sigma, United States) (Liong & Shah, 2005) were used. With a slight modification, 0.5% and 1% concentration of three bile salts were used. MRS broth was supplemented with 0.5% and 1 % (wt/vol) of oxgall, cholic acid or taurocholic acid. Before inoculation the cell density of the overnight grown fresh cultures isolates was maintained to an OD value of 1 at 600 nm for maintaining equal amount of bacteria in all tests. Broth was inoculated with each bacterium, and incubated at 30 °C MRS broth without bile salt was used as a control. Bacterial growth was monitored by measuring absorbance with a UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, United Kingdom) at 620 nm at 0 h, 4 h and 8 h respectively. Initial pH values of all the broths at time 0 were measured, and another measurement was taken after the final 8 h. All the experiments were replicated twice.

### 3.8.3. Cell Surface Hydrophobicity

Fresh cultures were grown in MRS broth at 30 °C for 24 h and centrifuged at 8,000 g for 5 min as per the method described by Rosenberg (1984). Cell density of all fresh cultures was maintained at OD value of 1 in 600 nm. The pellet was washed three times with Ringer solution (Merck, Germany), and thoroughly mixed in a vortex. 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 (HiMedia, India) in duplicates and mixed thoroughly in a vortex. The hexadecane and aqueous 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 cell surface hydrophobicity was expressed as follows:

$$\text{Cell surface hydrophobicity \%} = [(A_0 - A)/A] \times 100$$

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).

### 3.9. Cholesterol lowering mechanism of LAB

### 3.9.1. Screening of Cultures for Bile Salt Hydrolase (BSH) activity

The LAB isolates showing cholesterol lowering potential and other probiotic properties were further screened for BSH activity with slight modification. A loop full culture was taken and streaked on the MRS agar plates supplemented with 0.5% (w/v) TDCA (Sigma, United states). Plates were incubated anaerobically at 37 $^{\circ}$C for 72 h the amount of precipitation was visualized. MRS plates without supplementation were taken as controls. Isolates were tested in triplicates after which the presence of precipitated bile acid around colonies (opaque halo) or the formation of opaque granular white colonies with a silvery white shine was considered as a positive reaction (Dashkevicz et al., 1989; Ahn et al., 2003). The isolates that showed maximum precipitation were selected for further study. The isolates were grouped into two groups; Group I containing BSH positive isolates and Group II containing BSH negative isolates.

### 3.9.2. Assay for BSH enzyme

The BSH activity was determined as per method described by Tanaka et al. (2000). Overnight grown fresh cultures were grown in MRS broth for 20 h, then centrifuged at 10 000 g at 4 °C for 10 min. The bacterial pellet was washed twice and then suspended into 10 ml of 0.1M phosphate buffer (pH 7.0). The suspension was adjusted to an OD value of 1 unit at 600 nm. 5ml of the cell suspension was sonicated for 5 min (power level to 5, 30 sec on and 30 sec off) with constant cooling in ice, followed by centrifugation at 10 000 g at 4 °C for 10 min. Then to 0.1 ml of supernatant, 1.8 ml of 0.1M sodium phosphate buffer (pH 6) and 0.1ml of conjugated bile salt (6 mM sodium glycocholate (Sigma, United States) and 6 mM sodium taurocholate) were added (Sigma, United States). The mixture was incubated at 37 °C for 30 min. The enzymatic reaction was stopped by adding 0.5 ml of trichloroacetic acid (15% wt/vol) to 0.5 ml of sample. The mixture was centrifuged and to 0.2 ml of supernatant, 1ml of distilled water and 1ml of ninhydrin reagent was added. The solution was vortexed and then boiled for 14 min. Then the tubes were cooled and absorbance at 570 nm was determined using glycine or taurine as standards. A unit of BSH activity is defined as the amount of enzyme that liberates 1 µmol of amino acid from substrate per min. All experiments were repeated thrice.

### 3.9.3. Deconjugation of sodium glycocholate and sodium taurocholate

Deconjugation ability was determined as per protocol of Irvin et al. (1944) with little modifications. A 10ml volume of MRS broth was supplemented with 6 mM sodium glycocholate and 6 mM sodium taurocholate. Bacterial suspension was innoculated at 1% (v/v) and incubated anaerobically at 37 °C for 20 h. To 10 ml freshly grown culture after the incubation period was adjusted to pH 7.0 with NaOH (1 N). The cells

were centrifuged at 10 000 g (Micro CL 21R, Thermo Scientific, United States) at 4 $^{o}$C for 10 min. The supernatant was adjusted to pH 1.0 with HCl (1 N). Then to 1 ml suspension 2 ml of ethyl acetate was added and the mixture was vortexed for 1 min. 2 ml of the ethyl acetate layer was transferred into a glass tube and evaporated under nitrogen at 60 °C. The residue was immediately dissolved in 1ml of NaOH (0.01 N). After complete mixing, 1ml of 1% furfuraldehyde and 1ml of $H_2SO_4$ (16N) were added, and the mixture was vortexed for 1 min before heating at 65 $^{o}$C in a water bath for 10 min. The contents were cooled and to it 2 ml of glacial acetic acid was added and the mixture is vortexed for 1 min. Absorbance was read at 660 nm in UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, United Kingdom). The amount of cholic acid released was determined using cholic acid standard. All experiments were replicated thrice.

### 3.9.4. Co-precipitation of cholesterol

Freshly prepared sterile MRS broth was supplemented with 6 mM sodium glycocholate, 6 mM sodium taurocholate respectively. Polyoxyethanyl-cholesteryl sebacate (Sigma, United States) was filter sterilized and added to the broth at a final concentration of 70–100 µg/ml. The broth was inoculated with 1% (v/v) level of each overnight grown fresh isolate and incubated anaerobically at 37 $^{o}$C for 20 h. The water-soluble cholesterol had a solubility of 60 mg/ml and contained 30% cholesterol. Thus, initial cholesterol concentration varied between 70 to 100µg/ml and was quantified separately for every batch (Pereira & Gibson, 2002). After the incubation period, cells were centrifuged (10 000 g, 4 $^{o}$C, 10 min) and the remaining cholesterol concentration in the broth was determined using cholesterol estimating kit (Erba, Mannheim, Germany) by colorimetric method.

### 3.9.5. Incorporation of cholesterol into the cellular membrane

The incorporation of cholesterol into the cell membrane of the bacterial strains was studied according to the work described by Noh et al. (1997).

The MRS-THIO broth was prepared by supplementing MRS broth with 0.2% sodium thioglycolate. The broth was further supplemented with 0.3% oxgall. The broth media was then autoclaved for 15 min at 121 °C. The cholesterol used up was measured by preparing cholesterol-phosphatidylcholine micelles prepared according to Razin et al. (1974). Then 1ml cholesterol-phosphatidylcholine micelle was added to a tube containing 9 ml of MRS-THIO broth. Lipid vesicles were prepared from egg phosphatidylcholine (egg yolk lecithin, Sigma, United States) and soluble cholesterol (Sigma, United States) as follows; to chloroform liquid, phosphatidylcholine/ cholesterol in a molar ratio of 1.0:0.9 (solution of 22 mg phosphatidylcholine and 10 mg cholesterol) was added and the solution was dried under nitrogen gas ($N_2$) in a flask. After drying 10 ml of 0.4 M sucrose was added and the mixture was sonicated (three 15-min periods with 5-min interruptions) in the ice under $N_2$. The final vesicle suspension was centrifuged at 38 000 × g for 30 min at 4°C to remove metallic particles shed by the probe. The amount of lipid material settled during centrifugation was negligible. Over 95% of the lipid in the vesicle suspension was retained by dialysis. The vesicle suspension was kept at 4 °C and used within 2-3 days of its preparation.

The micelles prepared from Egg yolk-lecithin were stored in the refrigerator at 5 °C for further use. This mixture was used as an uninoculated control. The remaining 1% broth was inoculated (v/v) with a overnight culture and incubated at 37 °C for 24 h. After incubation, cells were removed by centrifugation at 12,000 x g and 4 °C for 10 min. The cholesterol testing kit (Erba, Maneinham, Germany) was used to

determine the amount of cholesterol in the spent broth and in the uninoculated control. Amount of cholesterol was calculated by using standard curve of Polyoxyethanyl-cholesteryl sebacate (Sigma, United States). The amount that was assimilated (µg/ml) by the cells was calculated by subtracting the amount in the spent broth from that in the uninoculated control.

### 3.9.6. Assimilation of cholesterol in presence of carbohydrates and lipid in media

The ability of the bacterial isolates to assimilate cholesterol was studied in the presence of both carbohydrates and lipid. Water soluble cholesterol was dissolved in 99% ethanol and Tween 80 mixed in 3:1 ratio at a final concentration in MRS broth is 70–100 µg/ml.

1% (v/v) freshly grown cultures were inoculated in MRS broth (Tween 80+dissolved cholesterol) and incubated at 37 °C for 24 h. The tubes were centrifuged at (12, 000 x g, 10 min, at 4 °C) in order to separate bacterial cells biomass and obtain clear MRS broth supernatant. The cholesterol concentration was assayed with the enzymatic diagnostic test kit (ERBA, Manenheim, Germany). The absorbance was measured in UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, United Kingdom).

Percentage value of cholesterol assimilation was calculated using the following formula

$$D = 100\text{-} (A - B)/C \quad x \ 100$$

where: A − cholesterol concentration in MRS broth containing suspension of bacterial cells and addition of cholesterol solution, B − cholesterol concentration in MRS broth containing bacterial cells suspension only, C − cholesterol concentration in MRS broth containing cholesterol solution only, D − cholesterol assimilation, % (Ziarno et al., 2007).

## 3.10. Phenotypic identification

Sugar fermentation tests of the selected and 14 identified strains were done using the carbohydrate fermentation kit, Hi Carbo$^{TM}$ Kit (Hi Media). The fresh overnight grown cultures were adjusted at an optical density of 0.5 O.D. at 620nm. Then 50μl culture was inoculated to 35 different wells, part A (lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L- arabinose and mannose) and part B1 (inulin, sodium gluconate, glycerol, salicin, dulicitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, A methyl D glucosidase) containing 12 each of carbohydrate utilization tests and part C for 11 carbohydrates (rhamnose, cellobiose, melizitose, α methyl mannoside, xylitol, ortho-Nitrophenyl-β-galactoside (ONPG), esculin, D- arabinose, citrate, malonate and sorbose). The plates were then incubated at 37 $^{o}$C for 18-24 h. Carbohydrate fermentation pattern were noted.

## 3.11. Genotypic identification

After successful screening of the isolates for probiotic property and phenotypic identification method, genotypic method of identification was done.

## DNA extraction

Total genomic DNA of selected strains of LAB was isolated by using the Genomic DNA isolation kit (Hi Media, India) as per the manufacturer's instructions.

## Polymerase chain reaction (PCR) of 16S rRNA gene

Most applications of molecular methods currently in use are based on characterization of ribosomal RNA (rRNA) genes or the rDNA. Two set of primers targeting bacterial 16S rRNA gene that is the universal bacterial primer 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3') (Liu et al., 2009) was used for amplification initially. Those isolates that could not be

amplified by the universal primer were reamplified using Leu F (5'-GTGCCTAATACATGCAAGTCG-3') and Leu R (5'-AGCTTCAAAGGTAGTCAAGAC -3') (Wang et al., 2016). GoTaq® Green Master Mix 2X (Promega, USA), a ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers, containing two dyes (blue and yellow) that allow visualization during electrophoresis was used. The 2X reaction mixture contained: upstream primer (10μM) 0.25–2.5μl (Conc 0.1–1.0μM), downstream primer (10μM) 0.25–2.5μl (Conc 0.1–1.0μM) DNA template 1–5μl, Template (10-25 ng) DNA 1.0 μl and the total volume was made up to 25 μl by adding nuclease free water. The thermo cycler was set for initial denaturation at 94 $^{o}$C for 5 min, (1 cycle), denaturation at 94 $^{o}$C for 1 min, annealing at 58 $^{o}$C for 1min, extension at 72 $^{o}$C for 2 min (35 cycles) and final extension at 72 $^{o}$C for 10 min (1 cycle) at 4 $^{o}$C for storage.

**Gel electrophoresis**

The DNA amplified was checked in agarose gel in 1.5% Ultrapure™ Agarose (HiMedia, India). The agarose was mixed with Tris-acetate-EDTA (TAE) electrophoresis buffer to the desired concentration, and heated in a microwave until completely melted. A dye, ethidium bromide (final concentration 0.5μg/ml) was added to the gel to facilitate visualization of DNA after electrophoresis. The gel was casted and then placed in the electrophoresis chamber. Samples containing DNA (10 μl) mixed with loading buffer (1μl) were then pipetted into the sample wells, the lid and power leads were placed on the apparatus, and a current was applied (Sambrook et al., 1989). 5 μL PCR products were analyzed by electrophoresis at 80 volts for 45 min. Gels were visualized by UV transillumination. PCR products were sequenced (Eurofins, Bangalore) and analyzed. FASTA nucleotide database query were used to

determine partial 16S rRNA sequences, to estimate the degree of similarity to other 16S rRNA gene sequences.

**Nucleotide sequence accession numbers**

The sequences of DNA were aligned. The 16s rRNA gene sequences of fourteen LAB strains were deposited in the National Center for Biotechnology Information (NCBI), nucleotide sequence databases under the accession numbers (KU601439 to KU601444, KX274030 to KX274035, KX354551 and KX387371).

**Phylogenetic analysis**

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches. The evolutionary distances were computed using the Kimura 2-parameter (Kimura, 1980). Evolutionary analyses were conducted in MEGA6.

**3.12. Antibiotic susceptibility test for *Enterococcus* sp. isolates**

Antibiotic susceptibility test was performed only for the *Enterococcus* sp. isolates.

**Preparation of test organisms:** The test organism for the study includes one Gram negative bacterium *Escherichia coli* and one gram positive bacterium *Staphylococcus aureus*. The test organisms were maintained at 4 $^{o}$C on nutrient agar slants. Active cultures for experiments were prepared by transferring loopful of bacterial culture in nutrient broth and incubating them at 37 $^{o}$C for 24 h.

**Preparation of bacterial suspension:** From each organism 3 to 5 colonies were picked from fresh agar plate and selected colonies were transferred to a tube containing 3-4µl of suitable nutrient broth. After proper mixing the broths were incubated at 37 $^{o}$C for 24 h. Turbidity was assessed by spectrophotometer. The

absorbance of the suspension showed range of 0.08-0.13 O.D at 625 nm equivalents to Mc Farland standard 0.5. Turbidity was adjusted by adding bacterial colony when the turbidity was too low and further incubating (Wiegand et al. 2008).

### 3.12.1. Antibiotic susceptibility test by disc diffusion method

The antimicrobial susceptibility was studied by using the method described by Bauer et al. (1966). MRS broth was inoculated with the isolates and incubated at 37 $^o$C for 24 h. Turbidity of the bacterial cultures was adjusted to 0.5 Mc Farland standards equivalent to cell density of $10^8$CFU/ml. The bacterial lawn was made on LSM agar using a sterile cotton swab. The streaking was done on the entire surface of the LSM agar plate thrice. The plate was turned $60^o$ between streaking to achieve uniform inoculation. The plates were allowed to dry for 10- 15 min. Using forceps the antibiotic discs were placed on the surface of LSM agar aseptically. Incubation was done at 37 $^o$C for 24 h – 48 h under anaerobic condition. After proper incubation the diameter of inhibition was measured using scale, results were expressed as sensitive (S, $\geq$ 21 mm), intermediate (I; 16-20 mm) and resistant (R; $\leq$ 15 mm) (Chartiers et al., 1998) with some exceptions; zone $\leq$ 19 mm for penicillin G is considered resistant, zone $\leq$ 14 mm for tetracycline and vancomycin, zone $\leq$ 13 mm, kanamycin and chloramphenicol are considered to be resistant (Chartiers et al., 1998). The tests organisms used were *E coli* MTCC 1089 and *Staphylococcus aureus* MTCC 7443 as control strains. The results obtained were compared to these two strains as published by the CLSI (2014).

### 3.12.2. Minimum Inhibitory Concentration (MIC) using MIC Strip

MIC determination paper strip coated with antibiotic in a concentration gradient manner, capable of showing MICs in the range of 0.016 µ/ml - 256 µ/ml of Hi Media was used. The preparation of inoculum for MIC was similar to those used in the disc

diffusion method. LSM agar plates were prepared for *Enterococcus* strains, Muller Hinton agar plates for *Staphylococcus aureus* and the nutrient agar plates for *E coli*. A sterile non-toxic cotton swab was dipped into the standardized inoculum and rotated firmly against the upper inside wall of the tube to express excess fluid. The entire agar plates were streaked with the swab three times turning the plate at 60 $^o$C between each streaking. The Ezy MIC $^{TM}$ (Hi Media, India) strip container was removed from the refrigerator and allowed to attain room temperature for 15 min before opening. One applicator from the sealed bag was removed; the applicator was held in the middle and gently pressed its broader sticky side on the centre of Ezy MIC $^{TM}$ strip. The strip was then placed on agar plate pre- spread with test culture. Care was taken to adjust the Ezy MIC $^{TM}$ strip at once and not to reposition.  The plates were incubated under anaerobic condition for 37 $^o$C for 24 h – 48 h for *Enterococcus* strains. The MIC was read where the ellipse intersects the MIC scale on the strip after incubation. The interpretation was made on the basis of the criteria for susceptibility categorization for each antibiotic.

# Results

## 4. Results

### 4.1. Documentation of traditional knowledge

Eastern Indian Himalayas highlights great diversity. This part of Himalayas has chilling cold as well as hot humid climate with wealthy flora and fauna (Gazetter of Sikkim, 2013). High topography hardly leaves any land for agriculture however this region is rich in medicinal plants. This differentiation in the ecosystems makes Sikkim a biodiversity hot spot region (Gazetter of Sikkim, 2013). Sikkim lies 27° 05' - 28° 09' north and 87° 59' - 88° 56' east covering an area of 7.096 km$^2$ and is one of the least populated Indian states surrounded by international borders of Tibetan plateau in the North, Nepal in the west, and Bhutan in the east and one national border that encloses the Darjeeling district of West Bengal in the South.  Sikkim has four districts; North, South, West and East colonized by a number of indigenous communities like Bhujels, Bhutias, Chettris, Dukpas, Gurungs, Kagateys (Yolmos and Sherpas), Kamis, Lepchas, Limbus, Lachenpas , Mangars, Mistris (Sarkis),  Newars, Rais,  Tamangs and Thamis residing in this site for at least the past several centuries (Gazetter of Sikkim, 2013).

The alpine temperate region in Sikkim is home to about 90% of the yak population of Sikkim. In the world domestic yak population is about 14.2 million and 71 000 yaks were reported to be present in India (Ramesha & Bhattacharya, 2008). There is 6468 yak in Sikkim according to the summary report on 18th livestock census 2007 of Sikkim state (Summary report on 18th Livestock census 2007, Sikkim State, Government of Sikkim).  After the twentieth century yak herding was transferred from Eastern Nepal to Khangchendzonga landscape originally inherited from Bhutan (Tambe & Rawat, 2009) and passed on to the Bhutias and Tibetian Dukpas. Yak is predominantly found at Lachen, Gurudongmar lake, Chopta valley, Lachung valley

and Yumthang in North district, Tsomgu Lake, Kupup, Thegu, Nathang in East district, and Yuksom-Dzongri in West Sikkim region. In the high altitude of Sikkim, yak rearing is the only alternative to provide sustainable source of income to the people of the alpine region. The local population herd yak known as *Dzomo* (cow-yak hybrid) in local language and domestic cattle. It is being raised by the herders for milk, meat and manure hence being referred to as a multipurpose animal. The herdsmen employ the traditional knowledge that were passed on from generations and have developed novel practices for yak husbandry and yak product technology.

In our study, the samples of the fermented milk products were collected from the 9$^{th}$ mile and Tsmogu lake region in the East District and Lachen and Lachung in North district of Sikkim as maximum yak populations are found in these regions. The households considered for study were basically those involved in yak rearing and its traditional practices in maintaining their livelihood. The yak management practices adopted, the processes involved in production of milk products and milk processing methods were studied in these areas. Open question was used for the collecting qualitative data among the respondents. During the survey, information on the benefits, purpose and research methodology were explained to them with the aim to preserve and conserve the traditional knowledge.

"Yak" is an English word derived from *gyag* in Bhutia language. Yak in English refers to both male and female but in Tibetan language, the male is called yak and female is called *De*. The yak and cattle hybrid is called *dzomo* (female) and *dzo* (male). Yak or *Bos gruinnens* belongs to class Mamalia and phylum Chordata, it has about 2 meters height and body covered with long hair that helps them to remain insulated in the cold alpine conditions of chilling Himalayas. It was suggested that the

**Figure 3:** Some Yak breeds in Sikkim

various body characteristics of yak is attributed as adaptations to extreme cold, high altitude with low oxygen content in the air and less nutrition grazing in herbaceous plants (Tambe & Rawat, 2009). Yak survives in the annual mean temperature below 5 $^o$C and the average in the hottest month is <13 $^o$C (Tambe & Rawat, 2009). It can survive cold temperatures up to -40 $^o$C and in 3000 – 5000 m altitude above the mean sea level. As per local traditional knowledge, its survival in such extreme condition is due to the presence of long coarse outer hair and an undercoat of fine hair on their bodies, and thick layer of fat deposited keep them insulated in such low temperatures. They adapt to low oxygen content due to the presence of a large chest, large lungs and a large heart relative to their overall body size. In North Sikkim it was observed that yaks differ in the amount in color their body, amount of hair, presence and absence of horns and amount of milk production (Figure 3). The yak raisers follow traditional method of managing yak owing to the topography and cultural influences. They maintain yak in a semi-migratory fashion. In extreme cold conditions in the winter there is decreased vegetation so yaks are moved to lower areas with better pasture and when the temperature starts to warm up in summer the yaks are migrated to higher altitudes into the temporary houses.

It was observed that yaks graze on short grass, creeping stems, roots and tender branches or shrubs and also graze on long grasses like other cattle do but they can also feed on short grasses.

The yak feeds on palatable shrubs like *Kobresia capillifolia* (*kesari buki*), *Kobresia napalensis* (*sun buki*),), *Phleum alpinum* (*doodhe jharr*), *Festuca valesiaca* (*rani buki*), *Carex nivalis* (*dharkhare), Kobresia duthiei* (*bhalu buki*), *Allium prattii* (*dandu*), *Heracleum* sp. (*ganer*), *Juncus* sp. (*suire buki*), *Selinum tenuifolium* (*cheeru*), *Rheum acuminatum* (*khokim*), *Carex nigra* (*harkat*), during the summer

**Table 3. Various ethnic milk products of yak milk**

| Local names of yak milk products | Common names in English |
|---|---|
| *Shyow* (Fermented) | Curd |
| *Khachu* (Fermented) | Whey |
| *Marr* | Butter |
| *Shuza/Shapjha or Phuicha* | Butter Tea |
| *Chhurpi* (Fermented) | Cheese |
| *Philu* | Creamy cheese |
| *Tema* | Cream |

season. These indigenous herbs besides having medicinal attributes are also considered by the herders to be nutrient rich (Tambe & Rawat, 2009). It was observed in some households that the herds are kept together and moved from less grazing pastures to more grazing pastures, but in some households yaks are kept in fenced areas where they are grazed in a surrounding area and taken to the shed for feeding. Apart from grazing yaks are fed with supplementary feed like molasses, barley, maize flour and water. The lactating herd, non- lactating herd and the pack herd are kept separately in the shed.

Yak hybrids produce more milk than pure yak. *Pheno*, a type of yak in Bhutia language gives less milk compared to *Hazi* type which gives more milk. Yak milk is thick in consistency, fatty, cream in color and has sweet in taste. Yak milk is rich in fat, protein, and healthy polyunsaturated fatty acids such as conjugated linoleic acid and omega-3 fatty acids and essential minerals, (Dong et al., 2007). Yak milk is

composed of 16.9- 17.7% solids, 4.9-5.3% protein, 5.5-7.2% fat, 4.5-5.0% lactose, and 0.8- 0.9% minerals (Lensch, 1996). During the lactation period of 180 days the yak milk yield is 147-487 kg (Lensch, 1996). The herders observe body weight of the adult body weight is 150 kg to 175 kg whereas young calves are about 10 kg to 16 kg. For milking the hind legs of yak are tied and then milking is started in the shed by women once during the early morning and in some cases twice also in the evening before the sun set when the cattle return from the pasture. The amount of milk driven by ranges from ½ liter to 2½ liter with the maximum milk yield during the months of August and September. Milk is kept without boiling in utensils made up of wood and sometimes plastic jars for several days. The milk collected at the expense of calf is preserved before being molded into various fermented products to earn their livelihood by the implementation of their indigenous knowledge.

Yak milk and its products are popular foods in high-altitude regions and it plays a major role in providing essential nutrients and minerals to the herdsman and to the local people. The most common Yak milk products in Sikkim are *Marr* (Ghew in Nepali) (Dewan & Tamang 2007), *Chhurpi* (Dewan & Tamang 2007), *Thara* (Mohi in Nepali), *Shyow* (Dahi in Nepali) (Dewan & Tamang 2007), *Chilu* (Yak Fat), *Philu* (Dewan & Tamang 2007) and *Tema* (Yak cream) (as per survey) (Table 3). Some yak milk products have been presented in Figure 4.

### 4.1.1. Yak milk fermented products

*Shyow*

In the local Bhutia language *Shyow* is known as *Dahi* (in Nepali) or curd. It is a popular fermented milk product of Sikkim Himalayas. It is a naturally fermented yak milk product or is prepared from the addition of a batch of a previously prepared

*Shyow*. It is often used for the preparation of other products like *Khachu* or *Mohi*, *Marr* or *Ghew* and soft *chhurpi* (Dewan &Tamang 2007).

### Khachu

*Khachu* refers to butter milk or whey in the local Bhutia language. It is known as *Mohi* in Nepali. It is obtained as a by-product of fermented *Shyow*. It is refreshing and a popular beverage of the alpine region.

### Marr

In the local Bhutia language Butter is known as *Marr*, it is known as *Ghew* in Nepali (Dewan and Tamang, 2007). It is one of the major yak milk products of the local community of alpine region of Sikkim. It is Rs 700- Rs 800/- per kg. The herder communities churn *the* curdled curd in wooden containers locally called as *zodong*. It is kept for consumption for cooking and frying edible items, or it is consumed directly. It is used to prepare *Tsamba* (barley) and also melted and added to barley and baked potato to be consumed as a snack by the local people. It is also used as a sacred offering for lighting lamps in *Gumpa* (Temple) for worshipping.

### Philu

Philu is prepared by adding fresh yak milk into cylindrical bamboo vessels (called *dzydung* by the Bhutia) or in wooden vessels (called *yadung*). On keeping the milk for a long period of time a soft creamy mass is formed and attached to the branches of a local plant that is kept in the vessel. After sufficient amount is formed it is then scraped off and stored as cheese having great value in the market (Dewan &Tamang, 2007).

**(a)**



**(b)**



**(c)**



**(d)**

**Figure 4**: The types of fermented yak milk products of Sikkim. (a) *Dongmo,* vessel for making *Marr*,    (b) *Marr* or Butter ready to be packed into leaf, (c) *Dudh Chhurpi,* (d) Soft *Chhurpi.*

*Chhurpi*

There are three different types of *Chhurpi*; hard type, soft type and *Dudh Chhurpi* (Figure 5). *Hard* type *Chhurpi* is made from yak milk that is boiled and a little whey is added to curdle causing the milk protein to get clumped together. After curdling it is strained in a cloth, this gives a soft texture then packed in a sack and pressed with heavy stones to remove water. It is then dried by hanging and eventually cut into cubical pieces. It costs about Rs 600/- per kg in the surrounding area and even more if marketed. Similarly, *Dudh Chhurpi* is like Hard *Chhurpi*, except milk is added after cutting it into cubical pieces and dried giving a white color and a rough texture. It is much expensive than Hard type *Chhurpi*. However, *Dudh Chhurpi* is much expensive than other two *Chhurpi.* The third type is Soft *Chhurpi* (Figure 4). It is soft *chhurpi* is prepared by boiling buttermilk or whey to form a white mass that is sieved out from the remaining liquid (Tamang, 2010). It is of soft texture and one of the most common traditional delicacies, consumed as a side dish or chutney being highly palatable and replaceable to even the non-vegetarian food among the Sikkimese community.

*Shuza/Shapjha* **or** *Phuicha*

The local people consume tea made up of yak milk with salt known as *Shuza/Shapjha* or *Phuicha* in the local language of the herders**.** It is believed to be one of the oldest traditional beverages of the indigenous community in the alpine region. They drink tea throughout the day providing the Tibetians the most possessed supplement in their diet. Salt tea has been most popular delicacy among the local people and consumed on

**Figure 5**: The processes involved in making Hard *Chhurpi:* (a) the curdled milk pressed with stones to obtain a hard block, (b) the hard blocked of chhurpi, (c) the hard block is cut into long pieces (d) and finally made into smaller square pieces.

a regular basis although sugar is available to them. In Nepali language it is called *Ghew Chea.* It is a refreshing beverage served by the local community to their guests.

## 4.1.2. Value added products from Yak Fiber

Two types of wool are found in yak, coarse outer hair and the inner soft hair coat called *Khullu* in Bhutia language. During shearing Yak gives more coarse hair of about 2 kg and 1/2 kg of fine hair. The hair of Yak is highly water proof with great tensile strength (Figure 6c).

The horns of Yak are also used for decorative purposes and considered holy. The horns are round and very thick about 15 - 20 cm in diameter. Fine wool or *Khullu* is used to make garments like muffler, sweaters and blankets. The fine wool woven to make garments resists rain and cold winter. Coarse hair is used to make tents, caps, blankets, hand bags, door mats and hand woven carpets. Carpets and door mats are woven in wooden frames traditionally and are used to cover the floor, chairs for making softer and warmer sitting in chairs. Good quality caps are also made by mixing the yak wool with the fine wool of Angora rabbit making them highly attractive, aesthetic and acceptable by the tourists. Ropes are prepared from long hairs that are twisted over to tie tents or domestic animals. The ropes are highly strong that can withstand sun, wind and rain. Slings locally known as *whurto,* made from coarse fibre is used to control yak herds and chase unwanted animals. Besides body hair yak tail also has religious value. The tail is washed properly and tied tightly with a rope in a wooded handle to make *Chamar* and used as a fly whisker in some areas in India. Besides a fly whisker it also has some aesthetic value. Its cost price in local markets ranges from Rs 5,000 to Rs 6,000 (Figure 6b). Yak skin is also used to make tents to resist cold, *mura* or stool (Figure 6a) and for many decorative purposes. Meat forms a major part of the diet of the local people in the North and Eastern part of Sikkim. The

local people prepare and consume a variety of traditionally processed smoked, sun-dried, air-dried, or fermented meat products, including sausages since olden days. Male yak is slaughtered instead of female yak. Mostly yak killed in accidents is consumed by the herdsman. The herdsmen consume yak meat on a daily basis and also during festivals like *Losar* in month of February (Figure 4d). Some of the yak meat products are *Satchu* (Dry meat), *Kargyong* or *Gyuma* (Sausages) (Figure 4e) and *Chilu* (Yak Fat). Yak *Satchu* costs Rs 500 to 600 per kg. According to one of the herdsman, in olden days fat separated from fresh meat was collected in sheep stomach, pressed with stones and hung in their houses but nowadays they use wooden jars to store fat until further use. Food cooked in its fat is considered as being tastier than in vegetable oil. Fat stored in these manner are used by the local community for a year or more.

Meat forms a major part of the diet of the local people in the North and Eastern part of Sikkim. The local people prepare and consume a variety of traditionally processed smoked, sun-dried, air-dried, or fermented meat products, including sausages since olden days. Male yak is slaughtered instead of female yak. Mostly yak killed in accidents is consumed by the herdsman. The herdsmen consume yak meat on a daily basis and also during festivals like *Losar* in month of February (Figure 6d). Some of the yak meat products are *Satchu* (Dry meat), *Kargyong* or *Gyuma* (Sausages) (Figure 6e) and *Chilu* (Yak Fat). Yak *Satchu* costs Rs 500 to 600 per kg. According to one of the herdsman, in olden days fat separated from fresh meat was collected in sheep stomach, pressed with stones and hung in their houses but nowadays they use wooden jars to store fat until further use. Food cooked in its fat is considered as being tastier than in vegetable oil. Fat stored in these manner are used by the local community for a year or more.

**Figure 6**: The multipurpose uses of yak fibre and yak meat, (a)Yak hair attached with skin to make *mura* or stool, (b) Yak tail or *Chamar,* (c) Yak fibre, drying of detached the skin with hair from the yak body, (d) Yak meat in normal drying process, (e) *Kargyong* or *Gyuma* (Sausages).

## 4.2. Isolation of lactic acid bacteria (LAB)

A total of 160 isolates from fermented Yak milk products namely *Shyow* (Dahi in Nepali)*, Marr* (Ghew in Nepali), *Thara* (Mohi in Nepali), *Chhurpi* of both soft and hard nature were collected from the Tsongmo lake region in the East District, Lachen and Lachung in North District of Sikkim were the study areas, as maximum yak populations are found in these regions. 60 human fecal isolates previously isolated were obtained from the Microbiology laboratory, Sikkim University. The isolates on the MRS agar plates were white, elevated colonies that were surrounded by a clear halo zone that were selected randomly and preserved (Figure 7). There were 160 Gram positive and catalase negative. Gram reaction was also confirmed by KOH test which was negative for all isolates. There were 70 rods and 90 cocci shaped isolates (Figure 8). The isolates were grouped as 150 homofermentative and 10 heterofermentative. All 160 isolates of fermented yak milk product and 60 cocci homofermentative fecal isolates were selected for screening for cholesterol lowering activity under *in vitro* condition.



**(a)**
**Figure 7**: Isolation of Lactic acid bacteria (LAB) on MRS agar with 1.5% CaCO$_3$
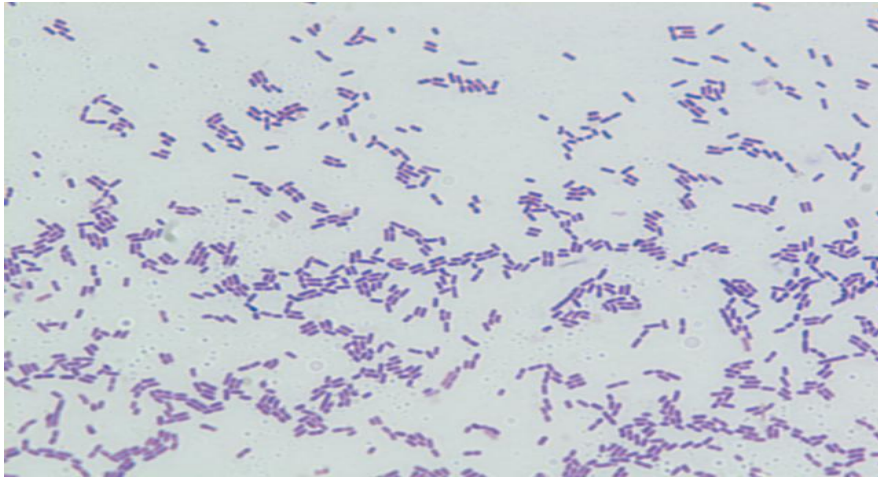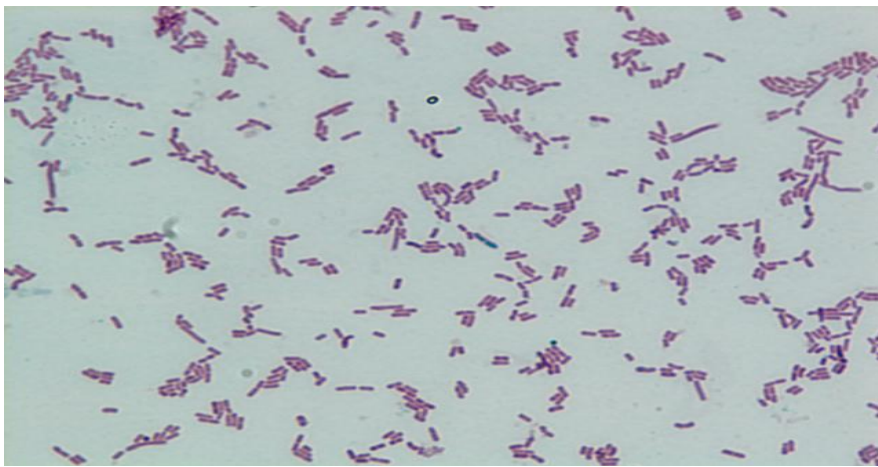
**Figure 8:** Morphology of some LAB isolates in compound microscope (Olympus, Japan) under oil immersion. (a) *Lactobacillus plantarum* YD5S, (b) *Lactobacillus plantarum* YD9S, (c) *Lactobacillus pentosus* YD8S
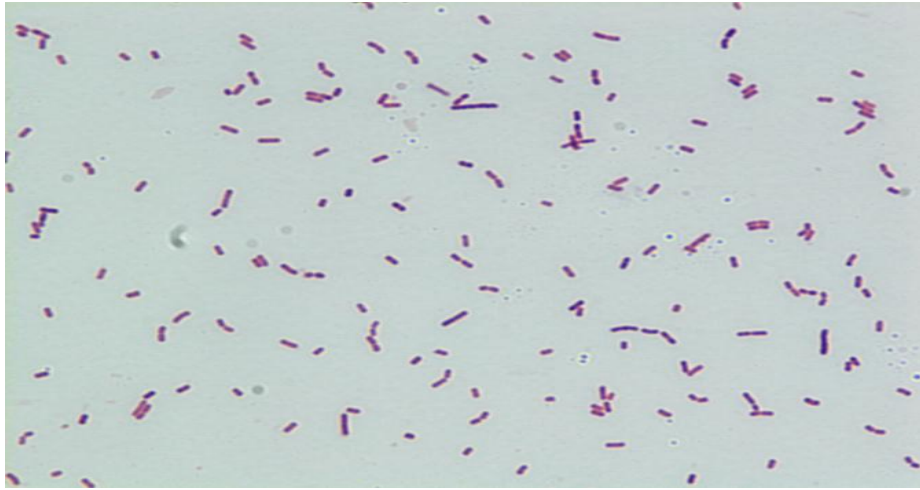
(d)
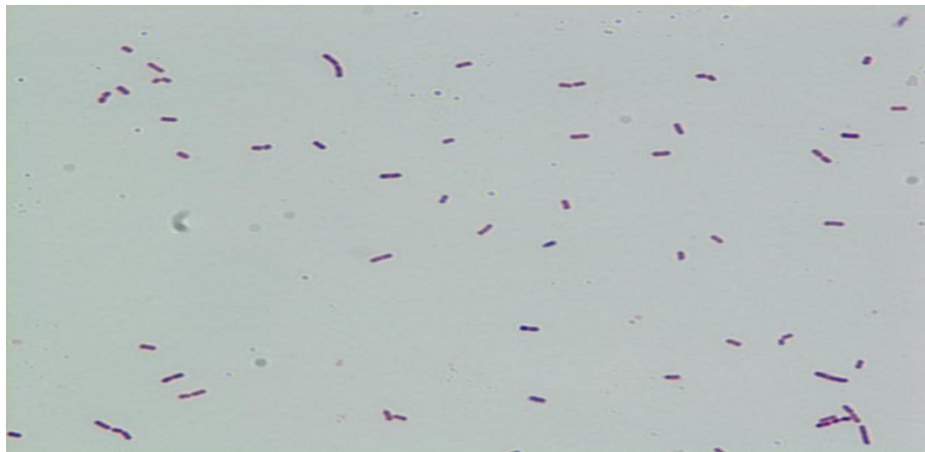


(e)
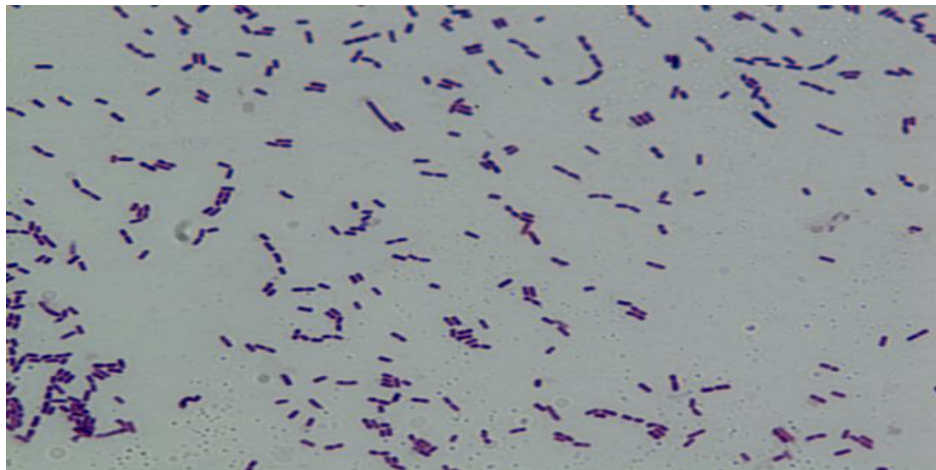


(f)

**Figure 8** continued: Morphology of some LAB isolates in compound microscope (Olympus, Japan) under oil immersion. (d) *Lactobacillus* YD15S, (e) *Lactobacillus* YHC3 (f) *Lactobacillus paraplantarum* YD11S
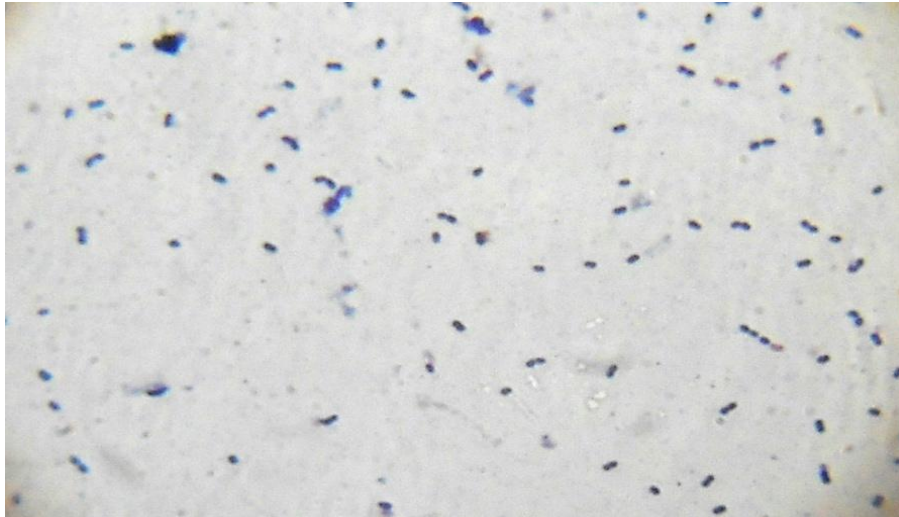
**(g)**
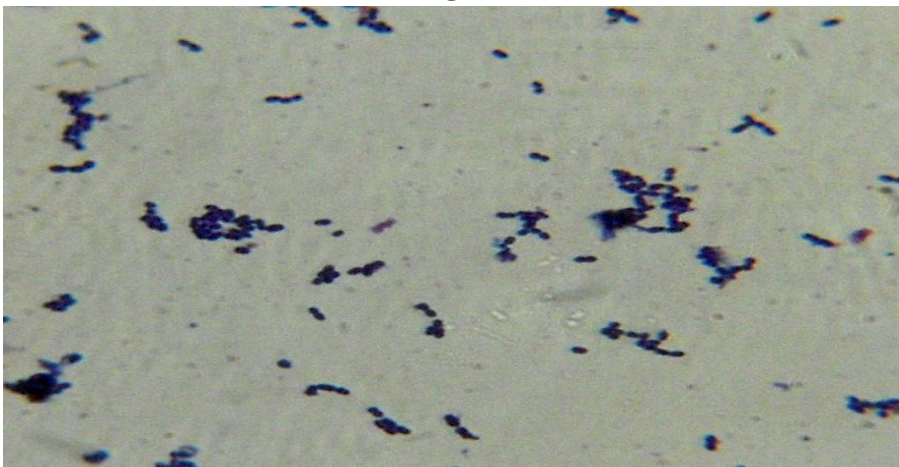


**(h)**
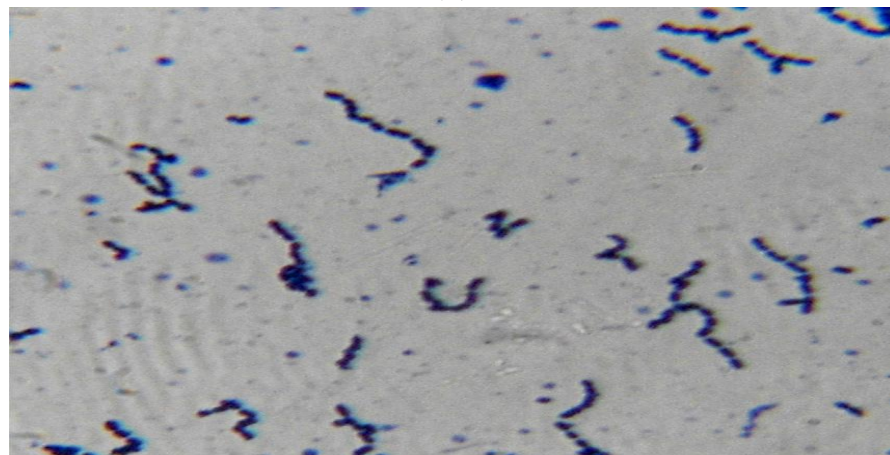


**(i)**

Figure 8 continued: Morphology of some LAB isolates in compound microscope (Olympus, Japan) under oil immersion. (g) *Enterococcus lactis* YY1, (h) *Enterococcus faecium* YHC20 (i) *Enterococcus faecium* HS31

## 4.3. Screening of LAB for cholesterol lowering

MRS broth supplemented with 70-100 µg/ml cholesterol inoculated with LAB cultures showed varying degrees of cholesterol lowering when compared to the un-inoculated control tube after 24hrs. 160 yak milk isolates and 60 human isolates were screened for cholesterol lowering under *in vitro* condition. The cholesterol lowering percentage was observed to be strain dependent in both the samples (Figure 9). 77% LAB isolates from fermented yak milk isolates showed cholesterol reduction of ≤ 50% and 23% isolates showed >50% reduction. Similarly, 43% human faecal isolates showed ≤ 50% reduction and 57% showed >50% reduction of in cholesterol. Percentage of cholesterol reduction of fermented yak milk product is presented in Table 4. The cholesterol lowering percentage ranged from 10% to 90% in comparison to the control tube. Maximum cholesterol removal activity was observed in *Pediococcus* YHC6  (90%), *Lactobacillus* YD5S (85%), *Lactobacillus* YW45 and *Pediococcus* YW35 (80%), *Lactobacillus* YHC12 (80%), *Enterococcus* YD12S (80%), *Lactobacillus* YD15S (75%), *Lactobacillus* YD9S (70%), and *Lactobacillus* YD7S (70%) strains. 35 isolates which showed cholesterol removal above 50% have been presented in Figure 10. Among the screening of 60 faecal isolates 33 isolates showed cholesterol lowering above 50%. The 33 cocci LAB isolates from human faeces showing more than 50% cholesterol reduction were selected for further study (Table 5). The isolates, *Enterococcus* HS03, *Enterococcus* HS06, *Enterococcus* HS08, *Pediococcus* HS22, *Enterococcus* HS23, *Enterococcus* HS29, *Enterococcus* HS40, *Enterococcus* HS43, and *Enterococcus* HS44 showed maximum cholesterol lowering activity of 70%. *Enterococcus* HS01,  *Enterococcus* HS02, *Enterococcus* HS04, *Enterococcus* HS09, *Enterococcus* HS10, *Lactococcus* HS17, *Enterococcus*
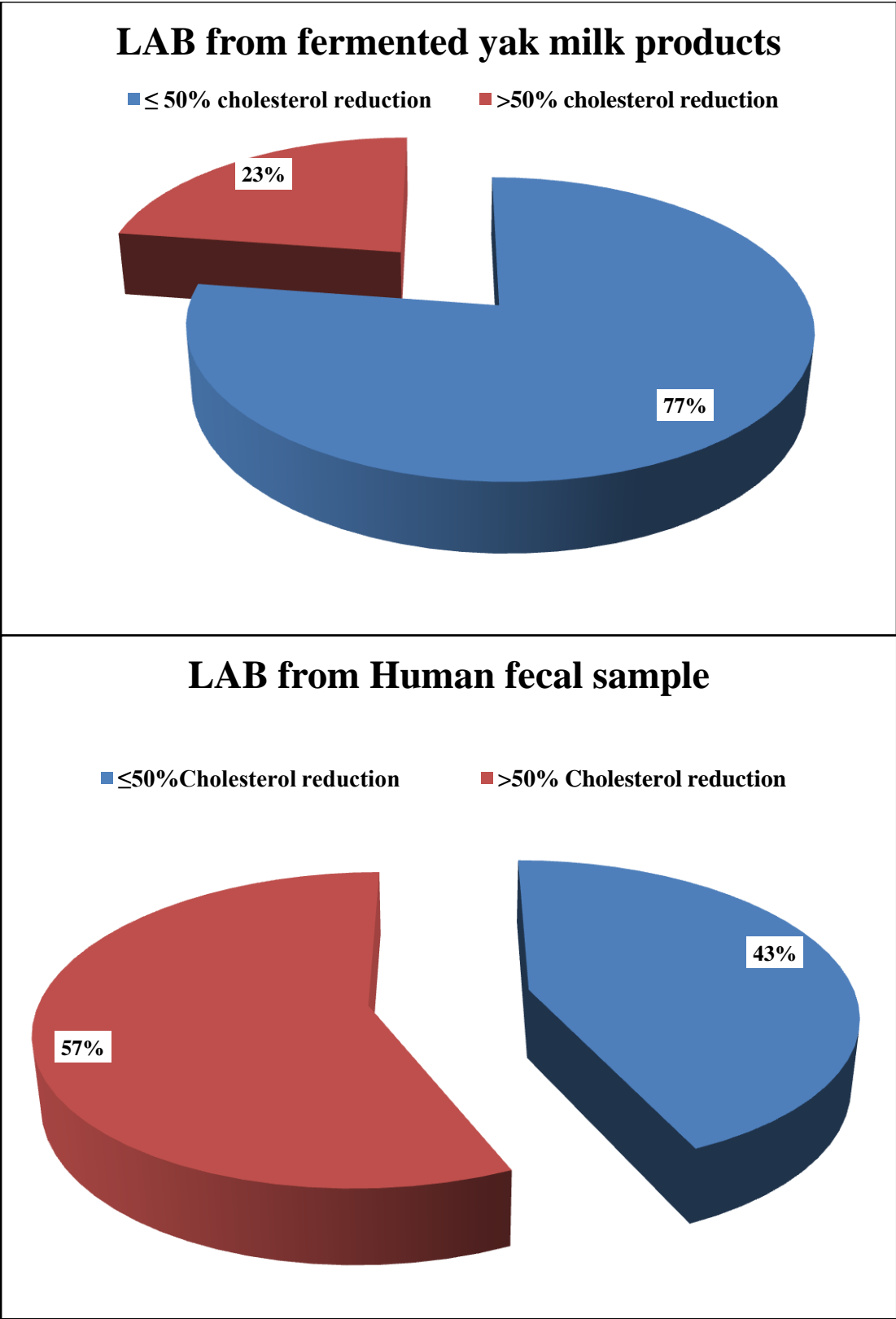
**Figure 9:** Cholesterol lowering percentages of LAB isolates from fermented yak milk products and human faecal samples

**Table 4:** Cholesterol reduction percentage by LAB isolates of fermented yak milk product

| Name of the product | Isolate code | Percentage of cholesterol reduction | Isolate code | Percentage of cholesterol reduction |
|---|---|---|---|---|
| *Thara* (Whey) n=33 | YW1 | 50±0.57 | YW18 | 25±1.73 |
| | YW2 | 25±0.57 | YW19 | 25±0.57 |
| | YW3 | 40±0.57 | YW20 | 40±1.73 |
| | YW4 | 20±0.57 | YW22 | 35±0.56 |
| | YW5 | 25±1.73 | YW23 | 25±0.57 |
| | YW6 | 65±0.60 | YW24 | 35±0.57 |
| | YW7 | 40±1.74 | YW25 | 10±0.57 |
| | YW8 | 40±0.57 | YW26 | 25±0.90 |
| | YW9 | 25±2.80 | YW27 | 40±0.80 |
| | YW10 | 50±0.50 | YW35 | 80±0.53 |
| | YW11 | 40±0.76 | YW41 | 65±0.75 |
| | YW12 | 40±0.10 | YW45 | 80±0.45 |
| | YW13 | 35±1.76 | YY1 | 65±0.43 |
| | YW14 | 35±0.57 | YC5 | 65±1.86 |
| | YW15 | 40±1.76 | YC5S | 65±1.45 |
| | YW16 | 50±0.55 | YC6S | 60±0.57 |
| | YW17 | 35±0.57 | | |
| Hard *Chhurpi* n=36 | YHC1 | 35±0.57 | YHC19 | 10±0.55 |
| | YHC2 | 45±1.75 | YHC20 | 65±0.57 |
| | YHC3 | 65±2.50 | YHC21 | 65±0.57 |
| | YHC4 | 65±0.57 | YHC22 | 30±1.55 |
| | YHC5 | 60±2.80 | YHC23 | 20±0.57 |
| | YHC6 | 90±0.57 | YHC24 | 25±0.57 |
| | YHC7 | 65±0.57 | YHC25 | 45±1.73 |
| | YHC8 | 50±1.75 | YHC26 | 35±1.75 |
| | YHC9 | 65±0.57 | YHC27 | 40±2.80 |
| | YHC10 | 65±1.75 | YHC28 | 20±2.50 |
| | YHC11 | 60±0.57 | YHC29 | 25±1.75 |
| | YHC12 | 80±0.57 | YHC30 | 30±0.54 |
| | YHC13 | 60±0.57 | YHC31 | 40±0.78 |
| | YHC14 | 40±0.57 | YHC32 | 40±1.75 |
| | YHC15 | 35±2.80 | YHC33 | 40±0.57 |
| | YHC16 | 45±0.57 | YHC34 | 40±0.57 |
| | YHC17 | 40±0.57 | YCH10 | 50±1.73 |
| | YHC18 | 25±2.80 | YCH4 | 65±1.73 |
| *Shyow* (Dahi) N=30 | YD1S | 35±2.50 | YD9S | 70±1.75 |
| | YD2S | 35±0.57 | YD10S | 35±0.57 |

**Table 4** continued: Cholesterol reduction percentage  by LAB isolates of fermented yak milk product

| Name of the product | Isolate code | Percentage of cholesterol reduction | Isolate code | Percentage of cholesterol reduction |
|---|---|---|---|---|
| *Shyow* (Dahi) n=30 | YD3S | 65±0.57 | YD12S | 80±0.57 |
| | YD4S | 25±0.50 | YD14S | 45±0.57 |
| | YD5S | 85±0.57 | YD15S | 75±1.80 |
| | YD6S | 60±0.57 | YD16S | 40±0.50 |
| | YD7S | 70±1.45 | YD17S | 25±0.56 |
| | YD8S | 75±0.57 | YD24S | 25±0.57 |
| | YD18S | 40±1.76 | YD25S | 10±0.57 |
| | YD19S | 30±0.57 | YD26S | 25±2.60 |
| | YD20S | 45±0.45 | YD27S | 35±1.75 |
| | YD21S | 40±1.75 | YD28S | 35±1.73 |
| | YD22S | 40±2.50 | YD29S | 35±1.45 |
| | YD23S | 15±0.57 | YD30S | 45±1.87 |
| | YD11S | 55±0.64 | | |
| *Marr (Ghew* or Butter*) n=26 | YGH1 | 40±0.57 | YGH14 | 20±0.57 |
| | YGH2 | 15±0.57 | YGH15 | 45±1.73 |
| | YGH3 | 25±0.57 | YGH16 | 15±1.09 |
| | YGH4 | 40±1.65 | YGH17 | 20±1.78 |
| | YGH5 | 40±0.57 | YGH18 | 20±0.50 |
| | YGH6 | 20±0.57 | YGH19 | 35±0.57 |
| | YGH7 | 15±1.50 | YGH20 | 25±0.57 |
| | YGH8 | 45±0.57 | YGH22 | 20±0.57 |
| | YGH9 | 40±2.30 | YGH23 | 45±1.10 |
| | YGH10 | 20±1.74 | YGH24 | 40±1.73 |
| | YGH11 | 40±2.80 | YGH26 | 35±0.57 |
| | YGH12 | 15±1.75 | YGH25 | 20±0.57 |
| | YGH13 | 40±1.60 | YGH27 | 35±0.68 |

The results are expressed as mean ± standard error of means; each data point is the average of three repeated measurements from two independently replicated experiments, n  indicates the no. of isolates from each samples

HS18, *Pediococcus* HS32, *Enterococcus* HS33, *Lactococcus* HS39 and *Enterococcus*

HS45 showed 65% cholesterol lowering percentage (Figure 11). A total of 68 LAB

isolates were therefore selected for further studies on identification, probiotic properties and the mechanisms of cholesterol lowering.

**Table 5:** Cholesterol reduction percentage of LAB isolates from human fecal sample

| Isolate code | Percentage of cholesterol reduction | Isolate code | Percentage of cholesterol reduction |
|---|---|---|---|
| HS01 | 65±0.57 | HS31 | 60±0.57 |
| HS02 | 65±1.73 | HS32 | 65±1.56 |
| HS03 | 70±0.57 | HS33 | 65±0.57 |
| HS04 | 65±1.35 | HS34 | 25±0.57 |
| HS05 | 60±0.60 | HS35 | 30±1.79 |
| HS06 | 70±0.57 | HS36 | 40±1.90 |
| HS07 | 25±0.57 | HS37 | 40±1.67 |
| HS08 | 70±0.57 | HS38 | 30±0.10 |
| HS09 | 65±0.57 | HS39 | 50±2.80 |
| HS10 | 65±1.15 | HS40 | 70±1.76 |
| HS11 | 35±2.89 | HS41 | 50±1.73 |
| HS12 | 60±0.20 | HS42 | 55±0.57 |
| HS13 | 50±1.73 | HS43 | 70±1.60 |
| HS14 | 45±0.57 | HS44 | 70±1.75 |
| HS15 | 50±1.50 | HS45 | 65±0.57 |
| HS16 | 30±0.50 | HS46 | 30±1.73 |
| HS17 | 65±1.56 | HS47 | 30±1.80 |
| HS18 | 65±1.75 | HS48 | 40±0.57 |
| HS19 | 60±1.45 | HS49 | 25±1.69 |
| HS20 | 55±1.45 | HS50 | 25±1.90 |
| HS21 | 55±1.75 | HS51 | 10±0.57 |
| HS22 | 70±1.56 | HS52 | 45±1.73 |
| HS23 | 70±0.57 | HS53 | 35±1.70 |
| HS24 | 55±0.57 | HS54 | 35±1.82 |
| HS25 | 50±1.73 | HS55 | 45±1.80 |
| HS26 | 50±1.45 | HS56 | 35±1.90 |
| HS27 | 45±0.57 | HS57 | 30±2.50 |
| HS28 | 45±0.57 | HS58 | 45±0.57 |
| HS29 | 70±1.50 | HS59 | 40±0.50 |
| HS30 | 45±0.57 | HS60 | 30±0.50 |

The results are expressed as mean ± standard error of means; each data point is the average of three repeated measurements from two independently replicated experiments
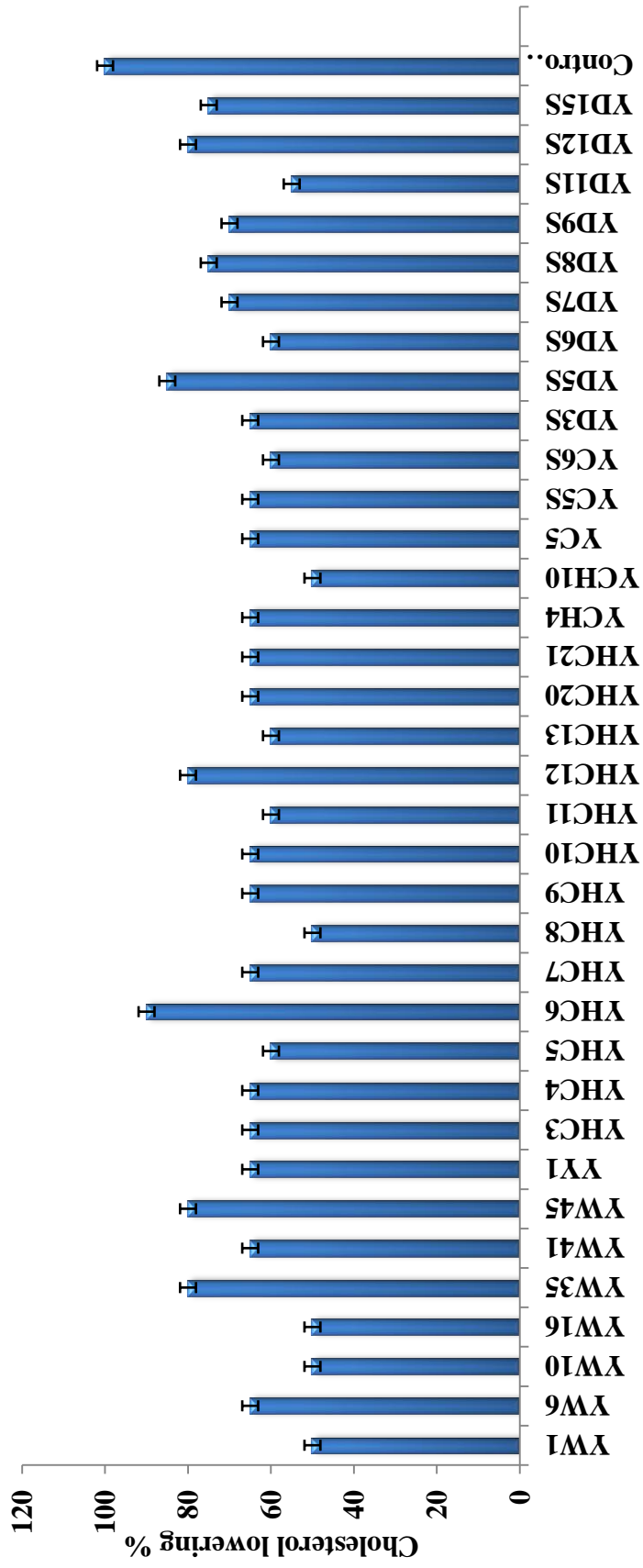
**Figure 10:** Cholesterol lowering activity of LAB isolates from fermented yak milk products. The control tube contained un-inoculated MRS broth with soluble cholesterol. Vertical line in each bar represents standard error.
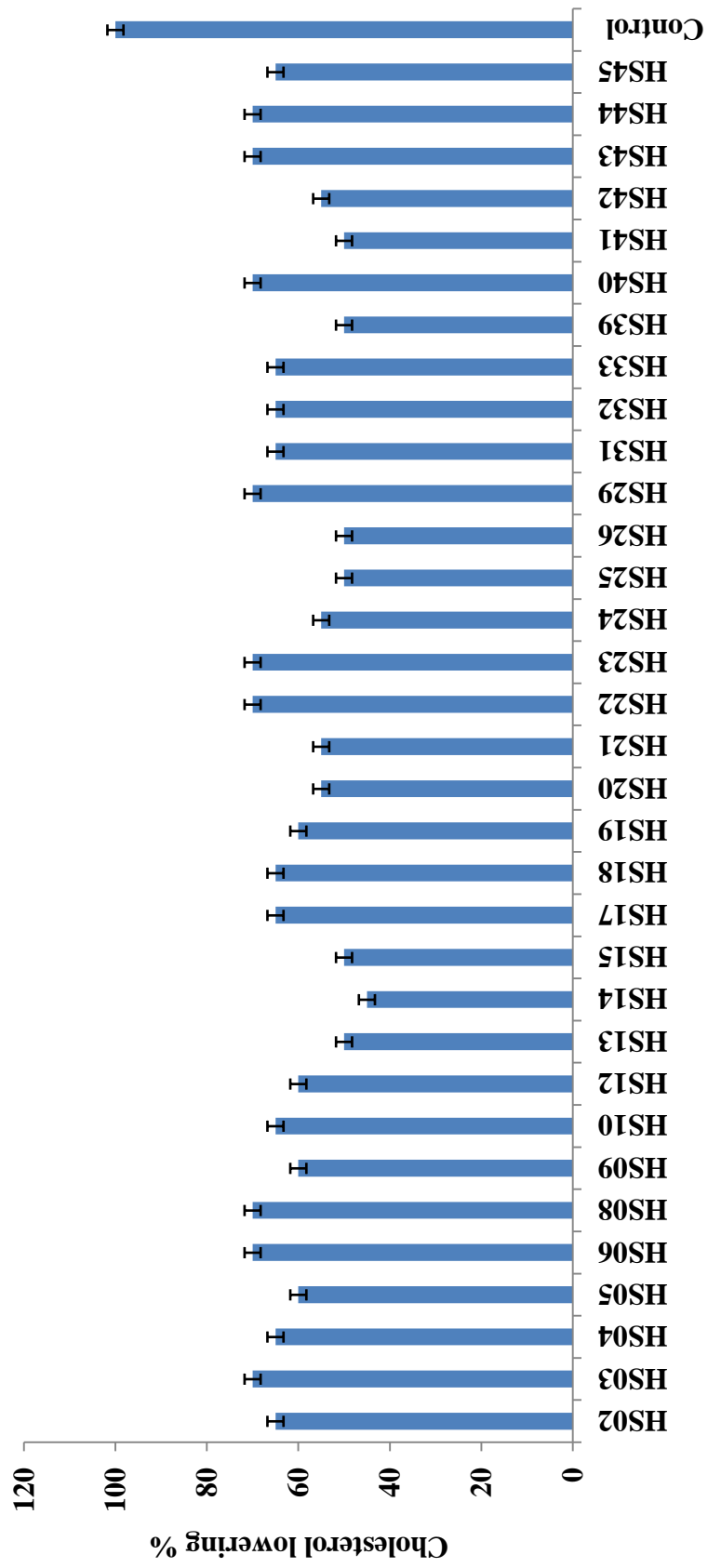
**Figure 11:** Cholesterol lowering activity of LAB isolates from human feces. The control tube contained un-inoculated MRS brothwith soluble cholesterol. Vertical lines in each bar represents the standard error.

## 4.4. Tentative identification

The rods and cocci shaped bacteria were segregated further depending on their growth patterns at different temperature, pH, salt concentration, arginine hydrolysis, $CO_2$ production from glucose and tetrad formation for classifying into different genera of lactic acid bacteria. In yak milk products genera belonging to *Lactobacillus* (46%), *Enterococcus* (28%), *Pediococcus* (20%) and *Lactococcus* (6%) were present; similarly the human faecal isolates contained *Enterococcus* (73%), *Lactococcus* (15%) and *Pediococcus* (12%) respectively. The tetrad cocci were directly placed under *Pediococcus* sp. The rod shaped, both gas forming and non gas forming isolates were placed under *Lactobacillus* sp. Similarly isolates were grouped as *Lactococcus* sp, and *Enterococcus* sp following the keys of Axelsson, 2004. There were no isolates belonging to *Tetragenococcus* or *Streptococcus*. Cocci-shaped LAB isolates growing at 6.5% NaCl and showing positive or negative growths were grouped into *Enterococcus* whereas those cocci isolates which were not able to grow in 6.5% NaCl were placed into *Lactococcus*.

The tentative identification of the Yak milk isolates and human faecal isolates are presented in Table 6 and Table 7 respectively.

## 4.5. Tolerance to low pH/Acid

The LAB selected for probiotic property, must be able to resist the high acidity that exists in the stomach in order to colonize and exert a positive effect. The pH of gastric juice secreted in the stomach is about 2 and many microorganisms are destroyed at this and lower pH. So, the tolerance to acidic conditions is a very important criterion for selection of probiotic bacteria (Huang &Adams, 2004).

**Table 6:** Phenotypic characters of LAB isolates from fermented yak milk products

| Source | Isolate Code | Arginine Hydrolysis | Tetrad formation | Shape | CO$_2$ production | Growth in/at 45°C | NaCl 6.5% | pH 4.4 | pH 9.6 | Genus |
|---|---|---|---|---|---|---|---|---|---|---|
| Khachu | YW1, YY1 | + | - | Coccus | - | + | + | 1/1 | + | *Enterococcus* |
| | YW16, YW41, YW45 | 2/1 | - | Rod | 1/2 | 2/1 | + | 1/2 | - | *Lactobacillus* |
| | YW10 | + | - | Coccus | - | - | - | + | - | *Lactococcus* |
| | YW6, YW35 | - | + | Coccus | - | 1/1 | 1/1 | + | - | *Pediococcus* |
| | YHC4, YHC5, YHC8, YCH10, YHC12, YHC13, YHC20, | + | - | Coccus | - | + | + | + | + | *Enterococcus* |
| Hard *Chhurpi* | YHC3, YHC11 | 1/1 | - | Rod | - | - | + | + | - | *Lactobacillus* |
| | YCH4 | + | - | Coccus | - | - | - | + | - | *Lactococcus* |
| | YHC6, YHC7, YHC9, YHC10, YHC21 | + | + | Coccus | - | 2/3 | + | + | - | *Pediococcus* |
| Soft *Chhurpi* | YC5, YC5S, YC6S | 1/2 | - | Rod | - | + | + | + | - | *Lactobacillus* |
| *Shyow* | YD12S | + | - | Coccus | - | + | + | + | + | *Enterococcus* |
| | YD3S, YD5S, YD6S, YD7S, YD8S, YD9S, YD11S, YD15S | 4/4 | - | Rod | - | 1/7 | + | + | - | *Lactobacillus* |

All isolates showed growth at temperature 10°C. +, all isolates positive; -, all isolates were negative, (... /..) number of positive isolates/ Number of negative isolates

**Table 7:** Phenotypic characters of LAB isolates from healthy human feces

| Isolate Code | Arginine Hydrolysis | Tetrad formation | $CO_2$ production from glucose | Growth in/at | | | | Genus |
|---|---|---|---|---|---|---|---|---|
| | | | | 45 °C | 6.5% NaCl | pH 4.4 | pH 9.6 | |
| HS02, HS03, HS04, HS05, HS06, HS08, HS10, HS12, HS13, HS18, HS21, HS23, HS24, HS25, HS26, HS29, HS31, HS33, HS40, HS41, HS42, HS43, HS44, HS45 | + | - | - | + | + | 11/13 | + | *Enterococcus* |
| HS09, HS14, HS17, HS20, HS39 | + | - | - | - | - | + | - | *Lactococcus* |
| HS15, HS19, HS22, HS32, | + | + | - | 1/3 | 2/2 | + | - | *Pediococcus* |

All were cocci, all showed growth at temperature 10℃,+, all isolates positive; -, all isolates were negative, (…/..), number of positive isolates/number of negative isolates

Acid tolerance of all 35 LAB isolates with cholesterol lowering properties was studied at a pH of 2.5 (Table 8). All isolates showed tolerance upto 1 h at pH 2.5 despite the variations in degree of viability. *Enterococcus* (YY1, YHC20, YHC12, YHC8, YD12S), *Lactobacillus* (YD8S, YD5S, YD15S, YD9S YD11S) and *Pediococcus* (YW35, YHC6) were most acid tolerant isolates with more than $10^3$ CFU/ml after incubation for 2 h at pH 2.5, while *Lactococcus* YCH10 and *Enterococcus* YW1showed $10^1$ total CFU/ml. The rest 21 isolates showed no survival at pH 2.5 after 2 h incubation. The 12 isolates which showed pH tolerance at 2.5 for 2 h were further tested at pH 2. The viable cell counts (log CFU/ml) and survival percentages of selected LAB to acid conditions at pH 2 after 2 h incubation are presented in Table 9. The isolate *Lactobacillus* YD8S and *Lactobacillus* YD9S have shown their maximum ability to tolerate the acidic pH for 2 h incubation. For *Lactobacillus* YD5S the counts decreased by 5.5 log cycles and for *Lactobacillus* YD9S the counts decreased by 5.83 log cycles, thereby showing greater acid tolerance over entire incubation period of 2 h. However in the rest of the 10 isolates of *Lactobacillus* (YD8S, YD11S and YD15S) showed greater acid tolerance and their counts decreased by 2.17-3.29 log cycles as compared to 6.47-7.15 log cycles for *Enterococcus* (YY1, YHC20). For acid sensitive isolates, the viability decreased slowly for the first hour of incubation followed by rapid decline at the end of 2 h incubation period. Similarly, tolerance to pH 2.5 was studied for 31 human faecal isolates till 2 h. Out of which 10 isolates was selected for further studies to check viability for 2 h at a pH 2. The effect of pH 2.5 on the growth of 33 LAB is presented in Table 10.The viability of isolates to tolerate acid was determined by their CFU/ml in 2h.

**Table 8**: Acid tolerance at pH 2.5 by LAB isolates from fermented yak milk products

| Genera | Viability count at pH 2.5 (log CFU/ml) | | |
|---|---|---|---|
| | **0 h** | **1 h** | **2 h** |
| *Enterococcus* YW1 | 9.50±0.12 | 4.60±0.45 | 1.02±0.23 |
| *Enterococcus* YY1 | 10.13±0.23 | 7.60±0.23 | 5.17±0.12 |
| *Enterococcus* YD12S | 9.45±0.48 | 6.54±0.39 | 4.15±0.29 |
| *Enterococcus* YHC4 | 8±0.12 | 3.11±0.11 | 0 |
| *Enterococcus* YHC5 | 8.79±0.15 | 4.24±0.45 | 0 |
| *Enterococcus* YHC8 | 10.05±0.13 | 7.61±0.34 | 3.28±0.12 |
| *Enterococcus* YHC12 | 10.40±0.11 | 8.36±0.18 | 4.20±0.10 |
| *Enterococcus* YHC13 | 7.89±0.21 | 3.24±0.18 | 0 |
| *Enterococcus* YHC20 | 9.89±0.28 | 7.84±0.11 | 5.89±0.24 |
| *Lactobacillus* YW16 | 10.11±0.54 | 5.02±0.32 | 0 |
| *Lactobacillus* YW41 | 9.20±0.12 | 3.20±0.24 | 0 |
| *Lactobacillus* YW45 | 8.25±0.34 | 2.01±0.35 | 0 |
| *Lactobacillus* YHC3 | 10.54±0.19 | 5.65±0.11 | 0 |
| *Lactobacillus* YHC11 | 7.68±0.24 | 3.12±0.59 | 0 |
| *Lactobacillus* YC5 | 9.85±0.29 | 3.02±0.45 | 0 |
| *Lactobacillus* YC5S | 8.21±0.12 | 3.10±0.11 | 0 |
| *Lactobacillus* YC6S | 8.10±0.37 | 4.28±0.49 | 0 |
| *Lactobacillus* YD3S | 8.25±0.18 | 2.89±0.28 | 0 |
| *Lactobacillus* YD5S | 10.13±0.23 | 7.60±0.11 | 5.50±0.14 |
| *Lactobacillus* YD6S | 8.61±0.11 | 2.50±0.45 | 0 |
| *Lactobacillus* YD7S | 8.79±0.27 | 3.15±0.48 | 0 |
| *Lactobacillus* YD8S | 10.35±0.09 | 7.02±0.27 | 5.63±0.25 |
| *Lactobacillus* YD9S | 11.01±0.37 | 8.85±0.16 | 5.50±0.11 |
| *Lactobacillus* YD11S | 10.01±0.23 | 7.45±0.11 | 5.05±0.15 |
| *Lactobacillus* YD15S | 10.40±0.16 | 8.36±0.19 | 4.20±0.28 |
| *Lactococcus* YW10 | 10.02±0.11 | 3.42±0.40 | 0 |
| *Lactococcus* YCH4 | 10.15±0.21 | 4.12±0.32 | 0 |
| *Lactococcus* YCH10 | 10.05±0.11 | 5.05±0.54 | 1±0.12 |
| *Pediococcus* YW6 | 9.23±0.15 | 3.21±0.23 | 0 |
| *Pediococcus* YW35 | 10.62±0.11 | 8.70±0.45 | 4.52±0.11 |
| *Pediococcus* YHC6 | 9.45±0.15 | 6.05±0.32 | 3.05±0.11 |

**Table 8** continued: Acid tolerance at pH 2.5 by LAB isolates from fermented yak milk products

| Genera | Viability count at pH 2.5 (log CFU/ml) | | |
|---|---|---|---|
| | 0 h | 1 h | 2 h |
| *Pediococcus* YHC7 | 8.25±0.23 | 3.02±0.21 | 0 |
| *Pediococcus* YHC9 | 8.10±0.27 | 4.25±0.37 | 0 |
| *Pediococcus* YHC10 | 8.01±0.34 | 5.68±0.23 | 0 |
| *Pediococcus* YHC21 | 8.01±0.09 | 2.05±0.23 | 0 |

Results are expressed as mean ± standard deviation of means; each data point is the average of two repeated measurements from two independently replicated experiments, n=2.

**Table 9**: Acid tolerance at pH 2 by selected LAB isolates from fermented yak milk products.

| Genera | Viability count at pH 2 (log CFU/ml) | | |
|---|---|---|---|
| | 0 h | 1 h | 2 h |
| *Enterococcus* YY1 | 9.67±0.007 | 3.2±0.01 | 0 |
| *Enterococcus* YHC8 | 8.6±0.05 | 0 | 0 |
| *Enterococcus* YHC20 | 9.36±0.004 | 2.45±0.007 | 0 |
| *Enterococcus* YD12S | 10.5±0.04 | 0 | 0 |
| *Lactobacillus* YHC12 | 9±0.07 | 0 | 0 |
| *Lactobacillus* YD5S | 9±0.005 | 6.56±0.007 | 3.5±0.007 |
| *Lactobacillus* YD8S | 11.29 ±0.001 | 8±0.01 | 0 |
| *Lactobacillus* YD9S | 9.028±0.002 | 5.54±0.05 | 3.2±0.01 |
| *Lactobacillus* YD11S | 11.17±0.05 | 9±0.05 | 0 |
| *Lactobacillus* YD15S | 10.76±0.01 | 8.3±0.05 | 0 |
| *Pediococcus* YW35 | 10.2±0.01 | 0 | 0 |
| *Pediococcus* YHC6 | 9.1±0.05 | 0 | 0 |

Results are expressed as mean ± standard error of means; each data point is the average of two repeated measurements from two independently replicated experiments, n=2.

**Table 10**: Acid tolerance at pH 2.5 by LAB isolates from human feces

| Genera | Viability count at pH 2.5 (log CFU/ml) | | |
|---|---|---|---|
| | **0 h** | **1 h** | **2 h** |
| *Enterococcus* HS02 | 8.20±0.30 | 4.68±0.40 | 1.25±0.11 |
| *Enterococcus* HS03 | 9.75±0.30 | 5.11±0.35 | 2.65±0.12 |
| *Enterococcus* HS04 | 10.13±0.25 | 8.60±0.28 | 5.20±0.35 |
| *Enterococcus* HS06 | 8.04±0.25 | 5.21±0.37 | 0 |
| *Enterococcus* HS08 | 10.18±0.20 | 7.10±0.50 | 3.50±0.33 |
| *Enterococcus* HS10 | 10.18±0.11 | 7.51±0.30 | 4.01±0.10 |
| *Enterococcus* HS12 | 9.01±0.20 | 3.50±0.33 | 0 |
| *Enterococcus* HS13 | 8.85±0.35 | 3.10±0.40 | 0 |
| *Enterococcus* HS18 | 10.50±0.11 | 6.30±0.40 | 2.70±0.11 |
| *Enterococcus* HS21 | 9.55±0.45 | 6.40±0.30 | 0 |
| *Enterococcus* HS23 | 10.01±0.30 | 8.68±0.23 | 4.50±0.30 |
| *Enterococcus* HS24 | 10.10±0.12 | 4.10±0.24 | 0 |
| *Enterococcus* HS25 | 8.5±0.45 | 3.45±0.20 | 0 |
| *Enterococcus* HS29 | 8.15±0.20 | 3.02±0.21 | 0 |
| *Enterococcus* HS31 | 10.25±0.10 | 6.25±0.50 | 3±0.15 |
| *Enterococcus* HS33 | 8.20±0.11 | 4.50±0.30 | 0 |
| *Enterococcus* HS40 | 9.24±0.10 | 4.50±0.10 | 0 |
| *Enterococcus* HS41 | 8.09±0.20 | 4.25±0.18 | 0 |
| *Enterococcus* HS42 | 9.40±0.10 | 6.36±0.10 | 0 |
| *Enterococcus* HS43 | 8.69±0.10 | 4.20±0.15 | 0 |
| *Enterococcus* HS45 | 8.11±0.15 | 3.50±0.15 | 0 |
| *Enterococcus* HS01 | 8.40±0.20 | 4.16±0.20 | 0 |
| *Enterococcus* HS44 | 10.15±0.30 | 8.05±0.15 | 4.50±0.10 |
| *Enterococcus* HS26 | 9.10±0.15 | 5.16±0.20 | 0 |
| *Enterococcus* HS05 | 8.45±0.11 | 3.50±0.52 | 0 |
| *Lactococcus* HS09 | 8.33±0.23 | 3.60±0.53 | 0 |
| *Lactococcus* HS14 | 8.15±0.20 | 4.10±0.37 | 0 |
| *Lactococcus* HS17 | 9.75±0.10 | 6.15±0.30 | 3.15±0.10 |
| *Lactococcus* HS20 | 8.70±0.25 | 3.45±0.40 | 0 |
| *Lactococcus* HS39 | 9.80±0.22 | 4.21±0.18 | 0 |
| *Pediococcus* HS15 | 8.10±0.19 | 4.10±0.10 | 0 |

**Table 10** continued: Acid tolerance at pH 2.5 by LAB isolates  human feces

| Genera | Viability count at pH 2.5 (log CFU/ml) | | |
|---|---|---|---|
| | **0 h** | **1 h** | **2 h** |
| *Pediococcus* HS19 | 9.05±0.17 | 6.25±0.27 | 0 |
| *Pediococcus* HS15 | 8.10±0.19 | 4.10±0.10 | 0 |
| *Pediococcus* HS19 | 9.05±0.17 | 6.25±0.27 | 0 |
| *Pediococcus* HS22 | 9.18±0.25 | 7.60±0.11 | 0 |

Results are expressed as mean ± standard error of means; each data point is the average of two repeated measurements from two independently replicated experiments, n=2.

**Table 11**: Acid tolerance at pH 2 by 10 selected LAB isolates from  human feces

| Genera | Viability count at pH 2(log CFU/ml) | | |
|---|---|---|---|
| | **0 h** | **1 h** | **2 h** |
| *Enterococcus* HS02 | 7.68±0.01 | 2.45±0.06 | 0 |
| *Enterococcus* HS03 | 9.60±0.34 | 7.01±0.12 | 3.0±0.31 |
| *Enterococcus* HS04 | 10.32±0.21 | 5.45±0.65 | 2.75±0.12 |
| *Enterococcus* HS08 | 9.12±0.54 | 2.45±0.14 | 0 |
| *Enterococcus* HS10 | 10.05±0.05 | 7.60±0.06 | 4.09±0.23 |
| *Enterococcus* HS18 | 10.07±0.04 | 8.01±0.03 | 4.01±0.02 |
| *Enterococcus* HS23 | 9.76±0.34 | 5.67±0.22 | 2.45±0.34 |
| *Enterococcus* HS31 | 10.12±0.23 | 6.23±0.43 | 2.65±0.02 |
| *Enterococcus* HS44 | 10.04±0.23 | 6.65±0.34 | 3.5±0.32 |
| *Lactococcus* HS17 | 8.10±0.12 | 4.03±0.11 | 0 |

Results are expressed as mean ± standard deviation of means; each data point is the average of two repeated measurements from two independently replicated experiments, n=2.

The isolates showed varying degrees of viability at pH 2.5 when incubated for 2h. *Enterococcus* (HS10, HS04, HS03, HS08, HS23, HS10, HS31 and HS02) and *Lactococcus* (HS17) were the most tolerant upto 2 h showing more than $10^2$ CFU/ml. The remaining isolates which did not show tolerance of pH 2.5 were not selected for pH 2 tolerance test. The tolerance of 10 isolates to pH 2 has been presented in Table 11. The isolates *Enterococcus* (HS10, HS03, HS44, HS18, HS23, HS31 and HS04) showed maximum tolerance at pH2. *Enterococcus* HS10 and HS18 showed minimum tolerance whose viability decreased by 5.96 log cycles

and 6.06 log cycles respectively within 2 h. Similarly *Enterococcus* (HS44, HS03, HS23 and HS31 showed 6.54 log cycles, 6.6 log cycles, 7.31 log cycles and 7.47 log cycles. The minimum tolerance was shown by *Enterococcus* HS04 where the count decreased by 7.7 log cycles.

## 4.6. Tolerance to bile acid

The effect of cholic acid, taurocholic acid and oxgall on the growth of twelve lactic acid bacterial isolates from fermented Yak milk products has been presented in Table 12a, 12b, 13a and 13b.   Oxgall was used as a source of both conjugated and deconjungated bile, cholic acid as deconjugated bile and taurocholic acid as a conjugated one, MRS broth without bile salt was used as a control in all the experiments. The percentage increase in turbidity/ tolerance and percentage decrease in pH after incubation of the broth cultures till 8h was calculated from the initial and final values. The more percentage increase in turbidity at 8h reflects better tolerance to that particular concentration of bile salt. The percentage increase in turbidity in MRS broth ranged from 326-1169%. The effect of 0.5 % of cholic acid, taurocholic acid and oxgall has been presented in Table 12. However the percentage decrease in pH was found not to be related to percentage increase in turbidity. The initial pH and the final pH of the media in presence and in absence of bile salts were monitored. Two different concentrations of bile salts were used. The decrease in pH of the media by different isolates was found to be isolate dependent. In 0.5% ox bile, percentage increase in turbidity ranged from 43- 242%. The highest percentage increase in turbidity was observed in *Enterococcus* YHC20 (242%), followed by *Lactobacillus* YD5S (210%), *Lactobacillus* YD8S (206%) and *Lactobacillus* YD9S (200%). The lowest tolerance was seen in YHC12

*Lactobacillus*. In 0.5% taurocholic acid the percentage increase in turbidity/ tolerance ranged from 341-1440%.

The highest percentage increase in turbidity or tolerance was shown by *Lactobacillus* YD8S (1440%), *Lactobacillus* YD5S (1158%), *Pediococcus* YW35 (1190%) with lowest tolerance observed in case of *Pediococcus* YHC6. Similarly the percentage increase in turbidity or tolerance in 0.5% cholic acid ranged from 36-581%, the highest percentage increase in turbidity or tolerance was observed in *Lactobacillus* YD15S and lowest in *Enterococcus* YHC8. The isolates presented better tolerance in taurocholic acid compared to other two bile salts. The pH of the broths monitored after 8 h showed varying degrees of percentage decrease in pH among isolates. The percentage decrease in pH was not related to percentage increase in turbidity or tolerance (Figure 13a, 13b, 13c).

In 1% ox bile the percentage increase in turbidity or tolerance ranged from 44 – 235% (Table 13). The highest tolerance was shown by *Lactobacillus* YD15S and lowest by *Enterococcus* YHC8. The percentage increase in turbidity or tolerance was in the range of 275 to 1356% in 1% taurocholic acid. The highest tolerance was shown by *Lactobacillus* YD8S (1356%), followed by *Lactobacillus* YD5S (1250%), *Lactobacillus* YD11S (1036%) and *Enterococcus* YD12S (1000%). The lowest tolerance was shown by *Enterococcus* YHC20. In maximum cases the growth of isolates has found to decrease in 1% bile salt. A comparison of the tolerance to three bile salts of concentration 0.5% and 1% has been presented with a bar graph (Figure 12a, 12b and 12c).

The effect of oxgall, taurocholic acid and cholic acid of LAB isolates from human faecal isolates at a concentration of 0.5% and 1% has been presented in table 14a, 14b, 15a and 15b. The increase in turbidity percentage in MRS broth

ranged from 730-1175%. At 0.5% oxgall, the increase in turbidity percentage ranged from 105- 308%. The highest percentage increase in turbidity was observed in *Enterococcus* HS31 (308%), *Enterococcus* HS02 (292%), *Enterococcus* HS03 (277%), *Enterococcus* HS22 (275%), *Enterococcus* HS44 (210%) and *Enterococcus* HS04 (200%). The lowest tolerance was seen in case of *Lactococcus* HS17 as 105%. In 0.5% taurocholic acid ranged from 606-1054%. The highest percentage increase in turbidity was shown by *Lactococcus* HS17 (1054%) and lowest by *Enterococcus* HS10 (606%). In case of 0.5 % cholic acid the range of highest percentage increase in turbidity was 56 to 210%. The highest percentage was shown by *Enterococcus* HS02 and lowest by *Enterococcus* HS04. The pH of the broth was monitored after 8h showed varying results depending on the isolates. The percentage decrease in pH was not related to the percentage increase in turbidity or tolerance (Figure 15a, 15b, 15c).

The percentage increase in turbidity at 1% cholic acid ranged from 109-331%. The highest percentage increase in turbidity was observed in *Enterococcus* HS18. In 1% taurocholic acid the highest tolerance was shown by *Enterococcus* HS23 (991%) and in 1% cholic acid the highest tolerance was shown by *Enterococcus* HS23 (191%). In 1% bile salt also the decrease in pH was not related to the increase in turbidity. A comparison of the tolerance to three bile salts of concentration 0.5% and 1% has been presented with a bar graph (Figure 14a, 14b and 14c).

**Table 12 a**: Bile tolerance of LAB isolates in different bile media 0.5% from fermented yak milk product

**Growth Media**

| Genera | MRS broth + 0.5% Ox bile | | | | | MRS broth + 0.5% Taurocholic acid | | | | | MRS broth + 0.5% Cholic acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time | | | pH | | Time | | | pH | | Time | | | pH | |
| | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 |
| *Enterococcus* YD12S | 0.10±0.20 | 0.17±0.12 | 0.27±0.32 | 6.67 | 6.43 | 0.12±0.01 | 0.20±0.05 | 1.07±0.21 | 6.67 | 5.65 | 0.12±0.12 | 0.14±0.12 | 0.23±0.20 | 6.74 | 6.49 |
| *Enterococcus* YHC8 | 0.17±0.05 | 0.20±0.01 | 0.25±0.21 | 6.67 | 6.58 | 0.14±0.03 | 0.44±0.03 | 1.4±0.03 | 6.67 | 5.54 | 0.11±0.11 | 0.12±0.11 | 0.15±0.01 | 6.74 | 6.60 |
| *Enterococcus* YHC20 | 0.19±0.13 | 0.34±0.22 | 0.65±0.03 | 6.67 | 6.43 | 0.18±0.40 | 0.45±0.02 | 1.60±0.23 | 6.67 | 5.51 | 0.12±0.21 | 0.45±0.20 | 0.55±0.01 | 6.74 | 6.10 |
| *Enterococcus* YY1 | 0.16±0.01 | 0.19±0.01 | 0.31±0.11 | 6.67 | 6.47 | 0.13±0.11 | 0.37±0.76 | 1.29±0.1 | 6.67 | 5.62 | 0.14±0.12 | 0.17±0.26 | 0.29±0.34 | 6.74 | 6.55 |
| *Lactobacillus* YD5S | 0.20±0.03 | 0.42±0.03 | 0.62±0.02 | 6.67 | 6.20 | 0.12±0.25 | 0.34±0.34 | 1.51±0.02 | 6.67 | 5.78 | 0.13±0.09 | 0.20±0.12 | 0.32±0.11 | 6.74 | 6.50 |
| *Lactobacillus* YD8S | 0.16±0.01 | 0.23±0.14 | 0.49±0.11 | 6.67 | 6.39 | 0.10±0.11 | 0.44±0.11 | 1.54±0.01 | 6.67 | 5.57 | 0.10±0.15 | 0.19±0.02 | 0.21±0.12 | 6.74 | 6.21 |
| *Lactobacillus* YD9S | 0.17±0.40 | 0.32±0.23 | 0.51±0.21 | 6.67 | 6.29 | 0.13±0.56 | 0.29±0.39 | 0.99±0.03 | 6.67 | 6.00 | 0.12±0.02 | 0.29±0.01 | 0.45±0.11 | 6.74 | 6.48 |

T0,T1, T2 values represent the mean of optical density/ turbidity at 0 h, 4 h and 8 h respectively , ± Standard error

Table 12a continued: Bile tolerance of LAB isolates in different bile media 0.5% from fermented yak milk products

**Growth Media**

| Genera | MRS broth + 0.5% Ox bile | | | | | MRS broth + 0.5% Taurocholic acid | | | | | MRS broth + 0.5% Cholic acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time | | | pH | | Time | | | pH | | Time | | | pH | |
| | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 |
| *Lactobacillus* YD11S | 0.10±0.31 | 0.16±0.14 | 0.25±0.76 | 6.67 | 6.47 | 0.10±0.02 | 0.20±01.1 | 1.06±0.34 | 6.67 | 5.64 | 0.10±0.16 | 0.12±0.23 | 0.19±0.16 | 6.74 | 6.35 |
| *Lactobacillus* YD15S | 0.21±0.23 | 0.31±0.21 | 0.43±0.12 | 6.67 | 6.60 | 0.12±0.13 | 0.37±0.23 | 0.40±0.3 | 6.67 | 6.0 | 0.11±0.65 | 0.15±0.25 | 0.68±0.02 | 6.74 | 6.10 |
| *Lactobacillus* YHC12 | 0.14±0.12 | 0.18±0.43 | 0.20±0.31 | 6.67 | 6.60 | 0.11±0.30 | 0.27±0.34 | 0.63±0.02 | 6.67 | 6.09 | 0.09±0.11 | 0.12±0.13 | 0.16±0.21 | 6.74 | 6.73 |
| *Pediococcus* YHC6 | 0.21±0.11 | 0.21±0.32 | 0.26±0.12 | 6.67 | 6.50 | 0.17±0.07 | 0.39±0.18 | 0.75±0.21 | 6.67 | 6.09 | 0.13±0.45 | 0.16±0.12 | 0.95±0.45 | 6.74 | 6.72 |
| *Pediococcus* YW35 | 0.16±0.13 | 0.18±0.10 | 0.36±0.40 | 6.67 | 6.45 | 0.11±0.11 | 0.33±0.02 | 1.42±0.32 | 6.67 | 5.66 | 0.11±0.11 | 0.32±0.11 | 0.27±0.11 | 6.74 | 6.48 |

T0=Initial Growth in 0h, T1= Growth in 4h, T2=Final in 8h, P1=Initial pH, P2 = Final pH, The bile tolerance measured as increase in optical density at 620nm.

**Table 12b**: Bile tolerance of LAB isolates in different bile media 0.5% from fermented yak milk products

| Genera | Growth media | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MRS broth | | MRS broth + 0.5% Ox bile | | MRS broth + 0.5% Taurocholic acid | | MRS broth + 0.5% Cholic acid | |
| | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH |
| *Enterococcus* YD12S | 807 | 14.15 | 170 | 3.59 | 791 | 15.29 | 92 | 3.71 |
| *Enterococcus* YHC8 | 782 | 16.61 | 47 | 1.35 | 900 | 16.94 | 36 | 2.10 |
| *Enterococcus* YHC20 | 735 | 16.15 | 242 | 3.60 | 788 | 17.39 | 358 | 9.50 |
| *Enterococcus* YY1 | 778 | 12.85 | 94 | 2.99 | 892 | 15.74 | 107 | 2.82 |
| *Lactobacillus* YD5S | 1169 | 18.15 | 210 | 7.50 | 1158 | 13.34 | 146 | 3.56 |
| *Lactobacillus* YD8S | 1150 | 14.76 | 206 | 4.19 | 1440 | 16.5 | 110 | 7.86 |
| *Lactobacillus* YD9S | 607 | 13.85 | 200 | 5.70 | 662 | 10.04 | 275 | 3.86 |
| *Lactobacillus* YD11S | 814 | 13.84 | 156 | 2.99 | 960 | 15.44 | 90 | 5.79 |
| *Lactobacillus* YD15S | 326 | 13.23 | 105 | 1.05 | 233 | 10.04 | 581 | 9.50 |
| *Lactobacillus* YHC12 | 479 | 10.62 | 43 | 1.05 | 473 | 8.7 | 78 | 0.15 |
| *Pediococcus* YHC6 | 395 | 12.62 | 136 | 2.54 | 341 | 8.7 | 631 | 0.29 |
| *Pediococcus* YW35 | 700 | 14.92 | 125 | 3.30 | 1190 | 15.14 | 145 | 3.86 |

**Table 13 a:** Bile tolerance of LAB isolates in different bile media 1% from fermented yak milk products

<table>
<tr><th rowspan="3">Genera</th><th colspan="15">Growth Media</th></tr>
<tr><th colspan="5">MRS broth + 1% Ox bile</th><th colspan="5">MRS broth + 1% Taurocholic acid</th><th colspan="5">MRS broth + 1% Cholic acid</th></tr>
<tr><th colspan="3">Time</th><th colspan="2">pH</th><th colspan="3">Time</th><th colspan="2">pH</th><th colspan="3">Time</th><th colspan="2">pH</th></tr>
<tr><th></th><th>T0</th><th>T1</th><th>T2</th><th>P1</th><th>P2</th><th>T0</th><th>T1</th><th>T2</th><th>P1</th><th>P2</th><th>T0</th><th>T1</th><th>T2</th><th>P1</th><th>P2</th></tr>
<tr><td>*Enterococcus* YD12S</td><td>0.18±0.12</td><td>0.20±0.19</td><td>0.26±0.03</td><td>6.67</td><td>6.51</td><td>0.10±0.22</td><td>0.22±0.02</td><td>1.10±0.03</td><td>6.67</td><td>5.64</td><td>0.13±0.45</td><td>0.19±0.35</td><td>0.22±0.06</td><td>6.74</td><td>5.78</td></tr>
<tr><td>*Enterococcus* YHC8</td><td>0.23±0.56</td><td>0.25±0.12</td><td>0.26±0.56</td><td>6.67</td><td>6.46</td><td>0.14±0.30</td><td>0.39±0.67</td><td>1.43±0.35</td><td>6.67</td><td>5.55</td><td>0.17±0.09</td><td>0.23±0.02</td><td>0.29±0.05</td><td>6.74</td><td>6.40</td></tr>
<tr><td>*Enterococcus* YHC20</td><td>0.14±0.45</td><td>0.23±0.10</td><td>0.35±0.54</td><td>6.67</td><td>6.51</td><td>0.12±0.23</td><td>0.20±0.18</td><td>0.45±0.59</td><td>6.67</td><td>6.00</td><td>0.14±0.03</td><td>0.25±0.07</td><td>0.3±0.32</td><td>6.74</td><td>6.43</td></tr>
<tr><td>*Enterococcus* YY1</td><td>0.17±0.02</td><td>0.25±0.19</td><td>0.33±0.30</td><td>6.67</td><td>6.5</td><td>0.13±0.59</td><td>0.36±0.56</td><td>1.40±0.03</td><td>6.67</td><td>5.65</td><td>0.12±0.04</td><td>0.14±0.06</td><td>0.22±0.08</td><td>6.74</td><td>6.82</td></tr>
<tr><td>*Lactobacillus* YD5S</td><td>0.16±0.37</td><td>0.26±0.01</td><td>0.45±0.47</td><td>6.67</td><td>6.31</td><td>0.14±0.23</td><td>0.50±0.11</td><td>1.89±0.04</td><td>6.67</td><td>5.10</td><td>0.10±0.90</td><td>0.22±0.04</td><td>0.30±0.03</td><td>6.74</td><td>5.99</td></tr>
<tr><td>*Lactobacillus* YD8S</td><td>0.15±0.01</td><td>0.24±0.23</td><td>0.43±0.04</td><td>6.67</td><td>6.31</td><td>0.11±0.11</td><td>0.44±0.01</td><td>1.60±0.01</td><td>6.67</td><td>5.46</td><td>0.10±0.03</td><td>0.19±0.08</td><td>0.22±0.06</td><td>6.74</td><td>6.35</td></tr>
<tr><td>*Lactobacillus* YD9S</td><td>0.19±0.23</td><td>0.20±0.06</td><td>0.39±0.99</td><td>6.67</td><td>6.52</td><td>0.16±0.15</td><td>0.34±0.04</td><td>1.50±0.03</td><td>6.67</td><td>5.50</td><td>0.11±0.09</td><td>0.31±0.08</td><td>0.45±0.04</td><td>6.74</td><td>6.31</td></tr>
</table>

T0,T1, T2 values represent the mean of optical density/ turbidity at 0 h, 4 h and 8 h respectively , ± Standard error

**Table 13 a** continued: Bile tolerance of LAB isolates in different bile media 1% from fermented yak milk product

Growth Media

| Genera | MRS broth + 1% Ox bile | | | | | MRS broth + 1% Taurocholic acid | | | | | MRS broth + 1% Cholic acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time | | | pH | | Time | | | pH | | Time | | | pH | |
| | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 |
| *Lactobacillus* YD11S | 0.15±13 | 0.18±0.23 | 0.24±0.09 | 6.67 | 6.54 | 0.08±0.04 | 0.16±0.12 | 0.91±0.03 | 6.67 | 5.76 | 0.09±0.54 | 0.11±0.57 | 0.12±0.05 | 6.74 | 6.50 |
| *Lactobacillus* YD15S | 0.20±0.09 | 0.26±0.01 | 0.67±0.45 | 6.67 | 6.0 | 0.13±0.29 | 0.27±0.16 | 0.68±0.05 | 6.67 | 6.20 | 0.16±0.09 | 0.38±0.04 | 0.41±0.09 | 6.74 | 6.20 |
| *Lactobacillus* YHC12 | 0.15±0.19 | 0.16±0.09 | 0.27±0.09 | 6.67 | 6.61 | 0.11±0.45 | 0.22±0.02 | 0.68±0.11 | 6.67 | 6.05 | 0.16±0.56 | 0.24±0.04 | 0.32±0.01 | 6.74 | 6.70 |
| *Pediococcus* YW35 | 0.16±23 | 0.21±0.34 | 0.37±0.14 | 6.67 | 6.50 | 0.11±0.23 | 0.27±0.09 | 1.20±0.09 | 6.67 | 5.70 | 0.11±0.07 | 0.20±0.34 | 0.21±0.03 | 6.74 | 6.71 |
| *Pediococcus* YHC6 | 0.22±0.16 | 0.23±0.07 | 0.29±0.10 | 6.67 | 6.60 | 0.17±0.34 | 0.26±0.05 | 0.69±0.03 | 6.67 | 6.05 | 0.15±0.45 | 0.24±0.02 | 0.30±0.02 | 6.74 | 6.60 |

T0=Initial Growth in 0h, T1= Growth in 4h, T2=Final in 8h, P1=Initial pH, P2 = Final pH, The bile tolerance measured as in increase in optical density at 620nm.

**Table 13b**: Bile tolerance of LAB isolates in different bile media 1% fermented yak milk product

| Genera | Growth media | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MRS broth | | MRS broth + 1% Ox bile | | MRS broth + 1% Taurocholic acid | | MRS broth + 1% Cholic acid | |
| | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH |
| *Enterococcus* YD12S | 807 | 14.15 | 44 | 2.39 | 1000 | 15.44 | 69 | 5.04 |
| *Enterococcus* YHC8 | 782 | 16.61 | 13 | 3.14 | 921 | 16.79 | 71 | 4.89 |
| *Enterococcus* YHC20 | 735 | 16.15 | 150 | 2.39 | 275 | 10.04 | 114 | 4.59 |
| *Enterococcus* YY1 | 778 | 12.85 | 94 | 2.54 | 977 | 15.29 | 83 | 1.18 |
| *Lactobacillus* YD5S | 1169 | 18.15 | 181 | 5.39 | 1250 | 23.53 | 200 | 11.12 |
| *Lactobacillus* YD8S | 1150 | 14.76 | 187 | 5.39 | 1356 | 18.14 | 120 | 5.78 |
| *Lactobacillus* YD9S | 607 | 13.85 | 105 | 2.24 | 838 | 17.54 | 309 | 6.37 |
| *Lactobacillus* YD11S | 814 | 13.84 | 60 | 1.94 | 1036 | 13.64 | 33 | 3.56 |
| *Lactobacillus* YD15S | 326 | 13.23 | 235 | 10.04 | 423 | 7.04 | 159 | 8.01 |
| *Lactobacillus* YHC12 | 479 | 10.62 | 80 | 0.89 | 518 | 9.29 | 100 | 0.59 |
| *Pediococcus* YHC6 | 395 | 12.62 | 32 | 1.04 | 306 | 9.29 | 100 | 2.07 |
| *Pediococcus* YW35 | 700 | 14.92 | 131 | 2.54 | 991 | 14.58 | 91 | 0.44 |
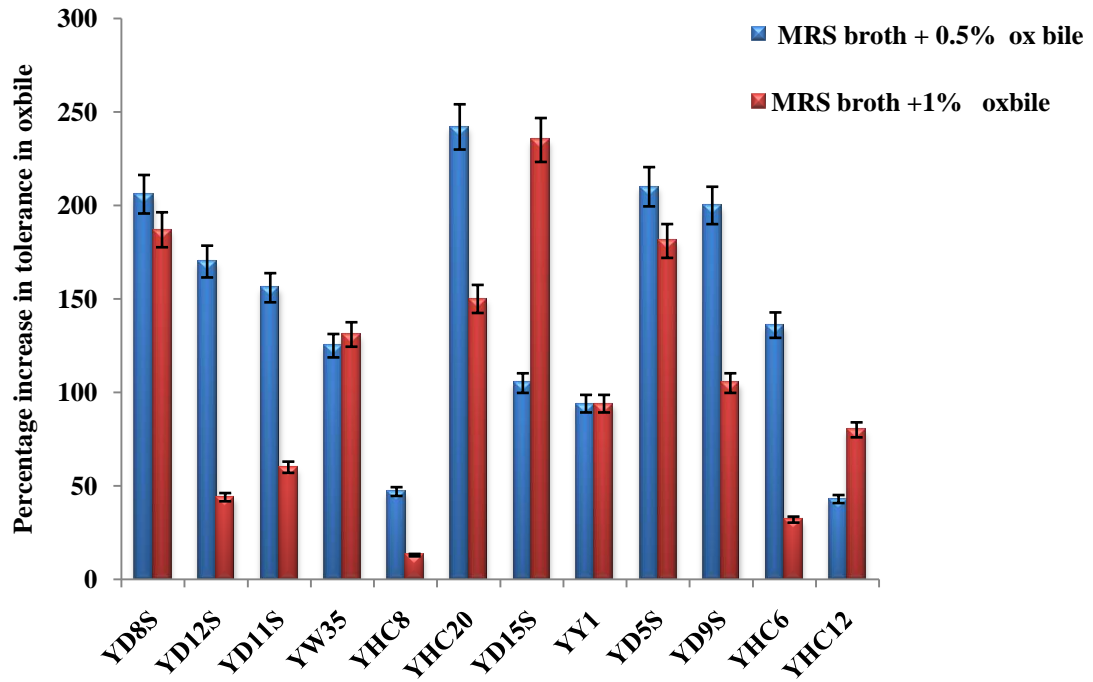
**Figure 12(a)**: Percentage increase in bile tolerance by LAB in 0.5% and 1% Ox bile after 8 h

Codes are *Lactobacillus* YD8S, *Enterococcus*YD12S, *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD15S, *Enterococcus* YY1, *Lactobacillus* YD5S, *Lactobacillus* YD9S, *Pediococcus* YHC6, *Lactobacillus* YHC12
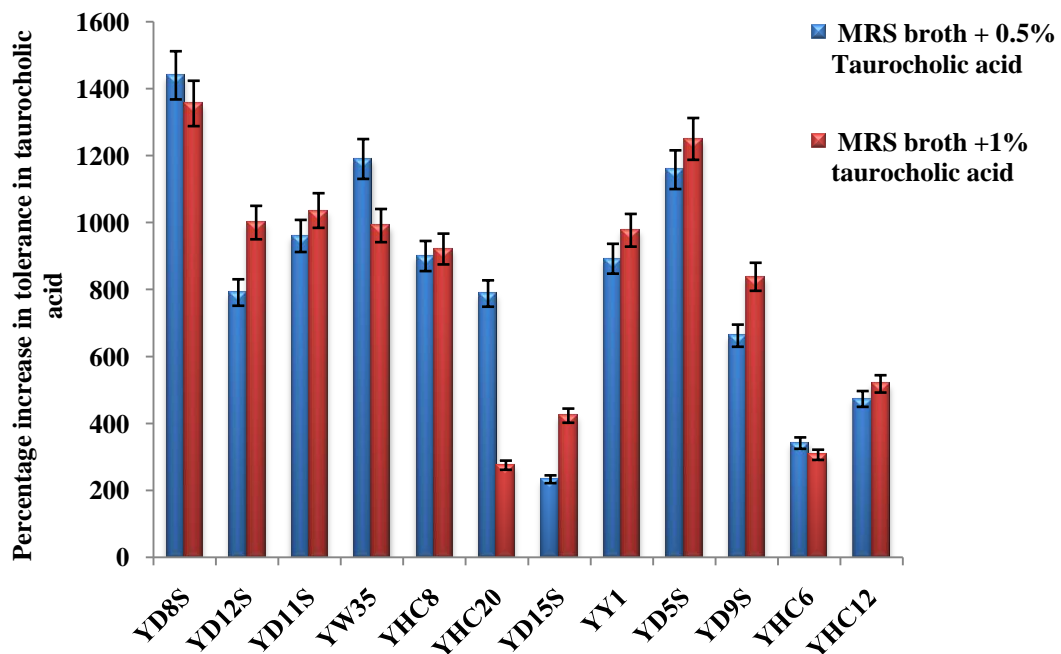


**Figure 12(b)**: Percentage increase in bile tolerance by LAB in 0.5% and 1% Taurocholic acid after 8 h

Codes are *Lactobacillus* YD8S, *Enterococcus*YD12S, *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD15S, *Enterococcus* YY1, *Lactobacillus* YD5S *Lactobacillus* YD9S, *Pediococcus* YHC6, *Lactobacillus* YHC12

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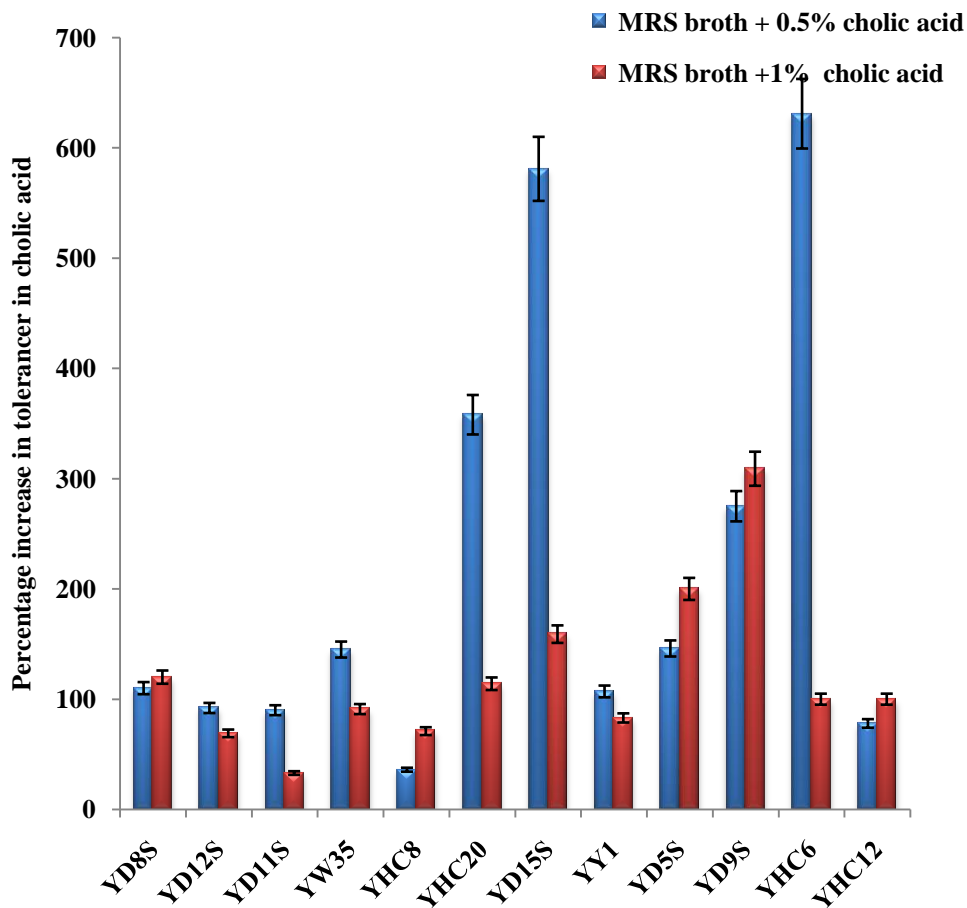
**Figure 12(c)**: Percentage increase in bile tolerance by LAB in 0.5% and 1% Cholic acid after 8 h.

Codes are *Lactobacillus* YD8S, *Enterococcus*YD12S, *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD15S, *Enterococcus* YY1, *Lactobacillus* YD5S *Lactobacillus* YD9S, *Pediococcus* YHC6, *Lactobacillus* YHC12
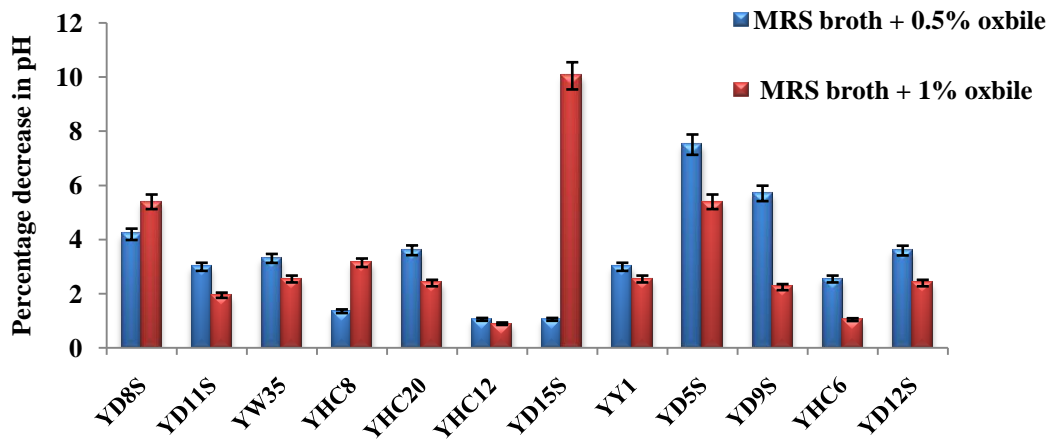
**Figure 13(a)**: Percentage decrease in pH by LAB in 0.5% and 1% Ox bile
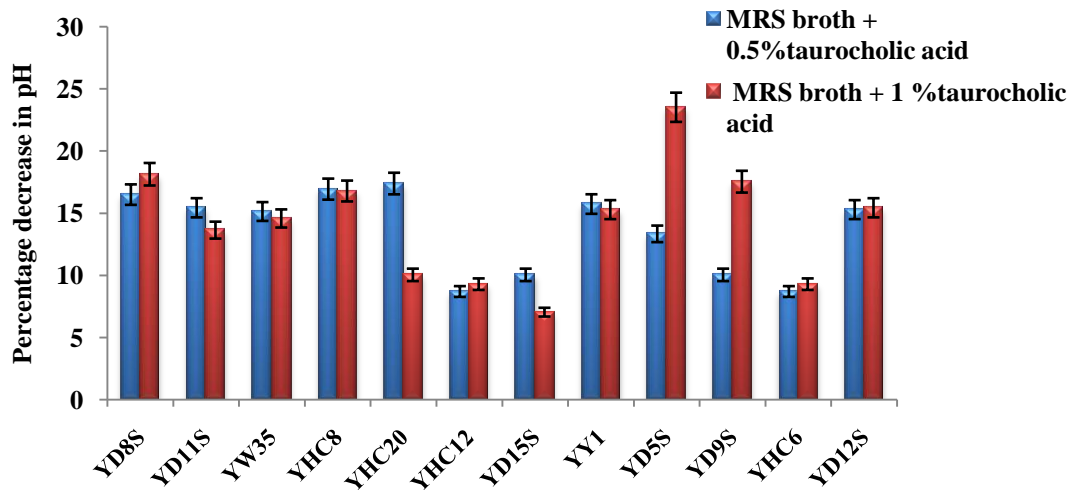


**Figure 13(b)**: Percentage decrease in pH by LAB in 0.5% and 1% taurocholic acid
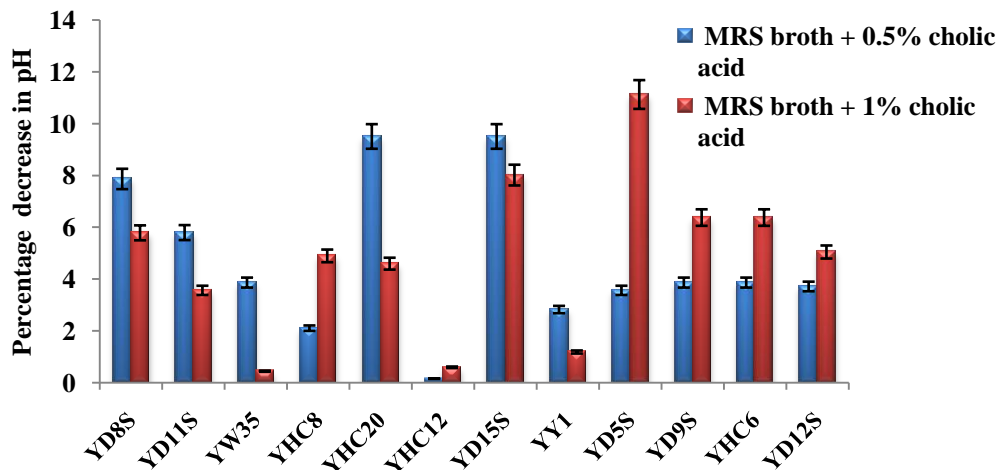


**Figure 13(c):** Percentage decrease in pH by LAB in 0.5% and 1% cholic acid

Note: Codes are *Lactobacillus* YD8S, *Enterococcus*YD12S, *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD15S, *Enterococcus* YY1, *Lactobacillus* YD5S *Lactobacillus* YD9S, *Pediococcus* YHC6, *Lactobacillus* YHC12.

**Table 14 a**: Bile tolerance of LAB isolates in different bile media 0.5% from human fecal sample

| | Growth Media | | | | | | | | | | | | | | |
| Genera | MRS broth + 0.5% Ox bile | | | | | MRS broth + 0.5% Taurocholic acid | | | | | MRS broth + 0.5% Cholic acid | | | | |
| | Time | | | pH | | Time | | | pH | | Time | | | pH | |
| | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Enterococcus* HS02 | 0.13±0.25 | 0.34±0.24 | 0.51±0.12 | 6.67 | 6.65 | 0.14±0.25 | 0.56±0.24 | 1.18±0.23 | 6.67 | 5.00 | 0.11±0.66 | 0.23±0.45 | 0.34±0.23 | 6.74 | 6.6 |
| *Enterococcus* HS03 | 0.13±0.23 | 0.33±0.34 | 0.49±0.45 | 6.67 | 6.00 | 0.14±0.12 | 0.47±0.11 | 1.56±0.23 | 6.67 | 5.09 | 0.13±0.11 | 0.15±0.23 | 0.21±0.25 | 6.74 | 6.7 |
| *Enterococcus* HS04 | 0.12±0.45 | 0.25±0.33 | 0.36±0.11 | 6.67 | 6.62 | 0.11±0.12 | 0.23±0.17 | 0.9±0.15 | 6.67 | 5.3 | 0.16±0.16 | 0.19±0.32 | 0.25±0.11 | 6.74 | 6.7 |
| *Enterococcus* HS08 | 0.23±0.54 | 0.33±0.23 | 0.59±0.19 | 6.67 | 6.65 | 0.13±0.12 | 0.45±0.11 | 1.45±0.13 | 6.67 | 5.21 | 0.11±0.02 | 0.23±0.09 | 0.33±0.23 | 6.74 | 6.6 |
| *Enterococcus* HS10 | 0.15±0.01 | 0.32±0.05 | 0.43±0.23 | 6.67 | 6.05 | 0.16±0.34 | 0.56±0.23 | 1.13±0.34 | 6.67 | 5.1 | 0.1±0.12 | 0.16±0.23 | 0.25±0.43 | 6.74 | 6.64 |
| *Enterococcus* HS18 | 0.19±0.22 | 0.34±0.17 | 0.41±0.19 | 6.67 | 6.6 | 0.16±0.02 | 0.55±0.29 | 1.23±0.21 | 6.67 | 5.67 | 0.11±0.23 | 0.17±0.22 | 0.27±0.22 | 6.74 | 6.71 |
| *Enterococcus* HS44 | 0.11±0.34 | 0.25±0.11 | 0.34±0.43 | 6.67 | 6.56 | 0.11±0.34 | 0.53±0.22 | 1.09±0.33 | 6.67 | 5.89 | 0.12±0.21 | 0.19±0.11 | 0.22 ±0.55 | 6.74 | 6.7 |
| *Enterococcus* HS23 | 0.12±0.11 | 0.23±0.34 | 0.45±0.23 | 6.67 | 6.6 | 0.17±0.09 | 0.65±0.08 | 1.35±0.13 | 6.67 | 5.21 | 0.13±0.34 | 0.26±0.11 | 0.35±0.12 | 6.74 | 6.6 |
| *Enterococcus* HS31 | 0.12±0.15 | 0.34±0.14 | 0.49±0.16 | 6.67 | 6.02 | 0.12±0.14 | 0.58±0.13 | 1.09±0.11 | 6.67 | 5.9 | 0.12±0.12 | 0.19±0.21 | 0.33±0.23 | 6.74 | 6.6 |
| *Lactococcus* HS17 | 0.21±0.56 | 0.33±0.25 | 0.43±0.24 | 6.67 | 6.03 | 0.13±0.24 | 0.33±0.23 | 1.5±0.45 | 6.67 | 5.5 | 0.14±0.24 | 0.17±0.25 | 0.25±0.23 | 6.74 | 6.7 |

T0,T1, T2 values represent the mean of optical density/ turbidity at 0 h, 4 h and 8 h respectively , P1=Initial pH, P2 = Final pH, the bile tolerance measured as in increase in optical density at 620nm. ± Standard error,

**Table 14 b**: Bile tolerance  LAB isolates in different bile media 0.5%  from human fecal sample

| Genera | MRS broth | | MRS broth + 0.5% Ox bile | | MRS broth + 0.5% Taurocholic acid | | MRS broth + 0.5% Cholic acid | |
|---|---|---|---|---|---|---|---|---|
| | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH |
| *Enterococcus* HS02 | 991 | 13.23 | 292 | 0.29 | 742 | 25.03 | 210 | 2.07 |
| *Enterococcus* HS03 | 1033 | 14.15 | 277 | 10.04 | 1014 | 23.68 | 62 | 0.59 |
| *Enterococcus* HS04 | 750 | 12.31 | 200 | 0.74 | 718 | 20.53 | 56 | 0.59 |
| *Enterococcus* HS08 | 1054 | 16.92 | 157 | 0.29 | 1015 | 21.88 | 200 | 2.07 |
| *Enterococcus*  HS10 | 990 | 13.23 | 187 | 9.29 | 606 | 23.53 | 150 | 1.48 |
| *Enterococcus* HS18 | 1170 | 13.08 | 116 | 1.04 | 669 | 14.99 | 145 | 0.44 |
| *Enterococcus* HS23 | 977 | 18.47 | 275 | 1.04 | 694 | 21.88 | 169 | 2.07 |
| *Enterococcus* HS31 | 942 | 13.85 | 308 | 9.74 | 808 | 11.54 | 175 | 2.07 |
| *Enterococcus* HS44 | 945 | 12.46 | 210 | 1.64 | 890 | 11.69 | 83 | 0.59 |
| *Lactococcus* HS17 | 1175 | 16.62 | 105 | 9.59 | 1054 | 17.54 | 79 | 0.59 |

Growth media

**Table 15 a:** Bile tolerance of LAB isolates in different bile media 1% from human fecal sample

**Growth Media**

| Genera | MRS broth + 1% Ox bile | | | | | MRS broth + 1% Taurocholic acid | | | | | MRS broth + 1% Cholic acid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time | | | pH | | Time | | | pH | | Time | | | pH | |
| | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 | T0 | T1 | T2 | P1 | P2 |
| *Enterococcus* HS02 | 0.14±0.34 | 0.34±0.78 | 0.45±0.23 | 6.67 | 6.32 | 0.11±0.25 | 0.29±0.34 | 0.40±0.12 | 6.67 | 6.32 | 0.12±0.09 | 0.21±0.29 | 0.27±0.18 | 6.74 | 6.70 |
| *Enterococcus* HS03 | 0.13±0.34 | 0.23±0.25 | 0.35±0.23 | 6.67 | 6.55 | 0.11±0.23 | 0.33±0.12 | 0.80±0.11 | 6.67 | 5.90 | 0.12±0.54 | 0.16±0.34 | 0.24±0.14 | 6.74 | 6.68 |
| *Enterococcus* HS04 | 0.11±0.46 | 0.21±0.45 | 0.30±0.35 | 6.67 | 6.59 | 0.15±0.25 | 0.39±0.23 | 1.12±0.09 | 6.67 | 5.21 | 0.15±0.56 | 0.24±0.45 | 0.34±0.34 | 6.74 | 6.60 |
| *Enterococcus* HS08 | 0.14±0.24 | 0.25±0.45 | 0.36±0.23 | 6.67 | 6.57 | 0.12±0.28 | 0.36±0.18 | 0.94±0.18 | 6.67 | 5.30 | 0.10±0.11 | 0.15±0.25 | 0.20±0.19 | 6.74 | 6.69 |
| *Enterococcus* HS10 | 0.12±0.34 | 0.25±0.02 | 0.33±0.23 | 6.67 | 6.56 | 0.16±0.09 | 0.34±0.12 | 0.60±0.13 | 6.67 | 5.80 | 0.11±0.14 | 0.15±0.17 | 0.23±0.09 | 6.74 | 6.70 |
| *Enterococcus* HS18 | 0.13±0.20 | 0.34±0.20 | 0.56±0.25 | 6.67 | 6.40 | 0.14±0.19 | 0.34±0.19 | 1.10±0.15 | 6.67 | 5.20 | 0.13±0.17 | 0.20±0.13 | 0.30±0.11 | 6.74 | 6.65 |
| *Enterococcus* HS23 | 0.11±0.09 | 0.19±0.12 | 0.23±0.21 | 6.67 | 6.60 | 0.11±0.12 | 0.34±0.20 | 1.20±0.21 | 6.67 | 5.00 | 0.11±0.54 | 0.21±0.45 | 0.32±0.34 | 6.74 | 6.56 |
| *Enterococcus* HS31 | 0.13±0.12 | 0.27±0.34 | 0.45±0.23 | 6.67 | 6.32 | 0.11±0.34 | 0.21±0.13 | 0.50±0.25 | 6.67 | 6.33 | 0.12±0.34 | 0.17±0.25 | 0.20±0.45 | 6.74 | 6.69 |
| *Enterococcus* HS44 | 0.12±0.25 | 0.25±0.23 | 0.34±0.54 | 6.67 | 6.56 | 0.12±0.12 | 0.21±0.11 | 0.90±0.06 | 6.67 | 5.30 | 0.11±0.25 | 0.18±0.15 | 0.20±0.26 | 6.74 | 6.69 |
| *Lactococcus* HS17 | 0.12±0.24 | 0.34±0.26 | 0.44±0.46 | 6.67 | 6.32 | 0.13±0.24 | 0.25±0.12 | 0.70±0.23 | 6.67 | 5.82 | 0.14±0.11 | 0.20±0.24 | 0.26±0.34 | 6.74 | 6.69 |

T0,T1, T2 values represent the mean of optical density/ turbidity at 0 h, 4 h and 8 h respectively , P1=Initial pH, P2 = Final pH, the bile tolerance measured as in increase in optical density at 620nm. ± Standard error

**Table 15 b:** Bile tolerance of LAB isolates in different bile media 1% from human fecal sample

| Genera | Growth media | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MRS broth | | MRS broth + 1% Ox bile | | MRS broth + 1% Taurocholic acid | | MRS broth + 1% Cholic acid | |
| | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH | % Increase in turbidity/ tolerance | % Decrease in pH |
| *Enterococcus* HS02 | 991 | 13.23 | 221 | 5.25 | 264 | 5.25 | 125 | 0.59 |
| *Enterococcus* HS03 | 1033 | 14.15 | 169 | 1.80 | 627 | 11.54 | 100 | 0.89 |
| *Enterococcus* HS04 | 750 | 12.31 | 173 | 1.20 | 647 | 21.89 | 127 | 2.08 |
| *Enterococcus* HS08 | 1054 | 16.92 | 157 | 1.50 | 683 | 20.54 | 100 | 0.74 |
| *Enterococcus* HS10 | 990 | 13.23 | 175 | 1.65 | 270 | 13.04 | 109 | 0.59 |
| *Lactococcus* HS17 | 1175 | 16.62 | 267 | 5.25 | 438 | 12.74 | 86 | 0.74 |
| *Enterococcus* HS18 | 1170 | 13.08 | 331 | 4.05 | 686 | 22.04 | 131 | 2.08 |
| *Enterococcus* HS23 | 977 | 18.47 | 109 | 1.05 | 991 | 25.04 | 191 | 2.67 |
| *Enterococcus* HS31 | 942 | 13.85 | 246 | 5.25 | 355 | 5.10 | 67 | 0.74 |
| *Enterococcus* HS44 | 945 | 12.46 | 183 | 1.65 | 650 | 20.54 | 82 | 0.74 |
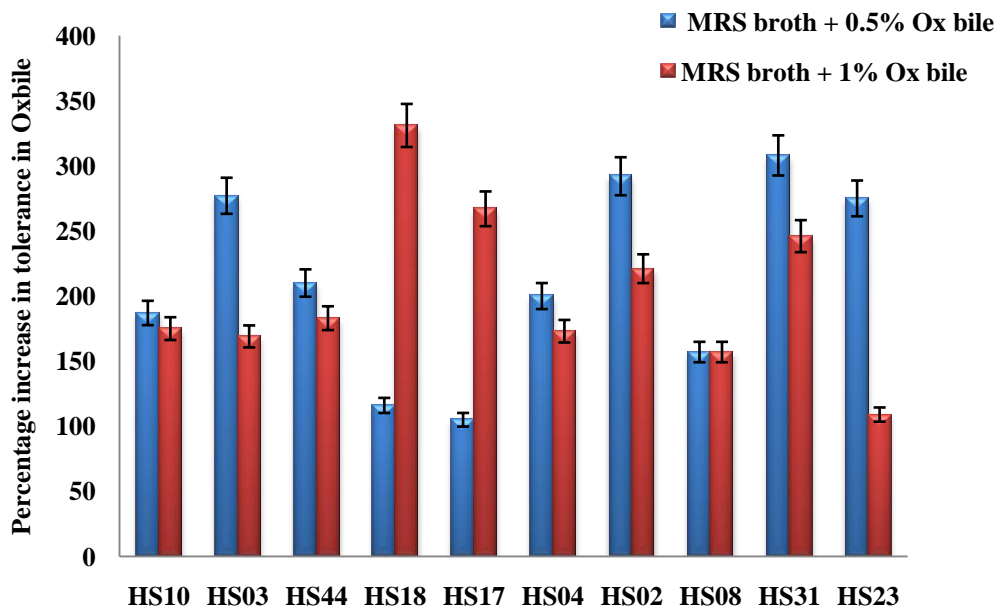
**Figure 14(a):** Percentage increase in bile tolerance by LAB in 0.5% and
1% Ox bile after 8h

The codes are *Enterococcus* HS1, *Enterococcus* HS03, *Enterococcus* HS44, *Enterococcus* HS18, *Lactococcus* HS17, *Enterococcus* HS04, *Enterococcus*HS02, *Enterococcus* HS08, *Enterococcus* HS31, *Enterococcus* HS23.
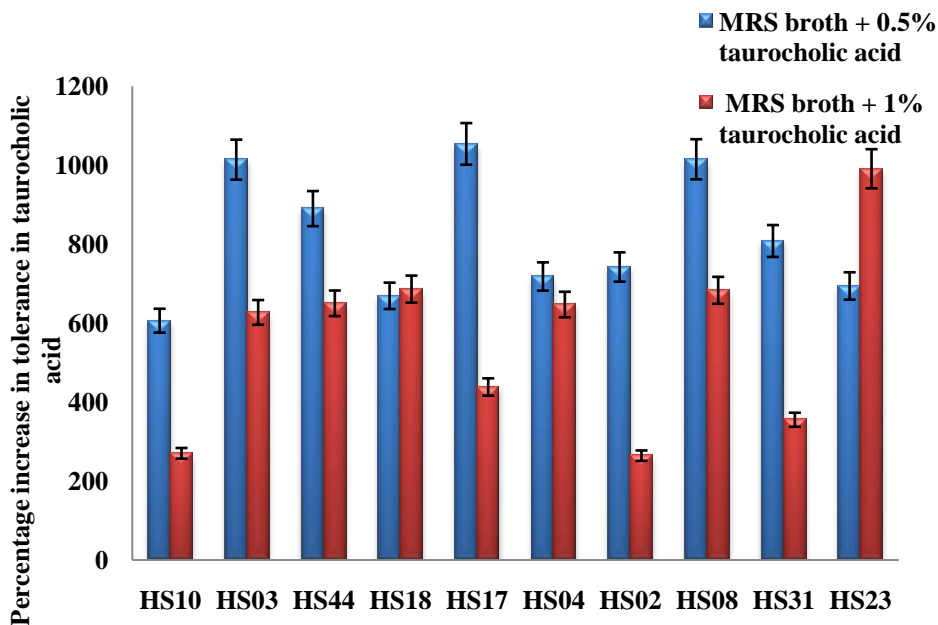


**Figure 14(b):** Percentage increase in bile tolerance by LAB in 0.5% and
1% Taurocholic acid after 8h

The codes are *Enterococcus* HS1, *Enterococcus* HS03, *Enterococcus* HS44, *Enterococcus* HS18, *Lactococcus* HS17, *Enterococcus* HS04, *Enterococcus*HS02, *Enterococcus* HS08, *Enterococcus* HS31, *Enterococcus* HS23.

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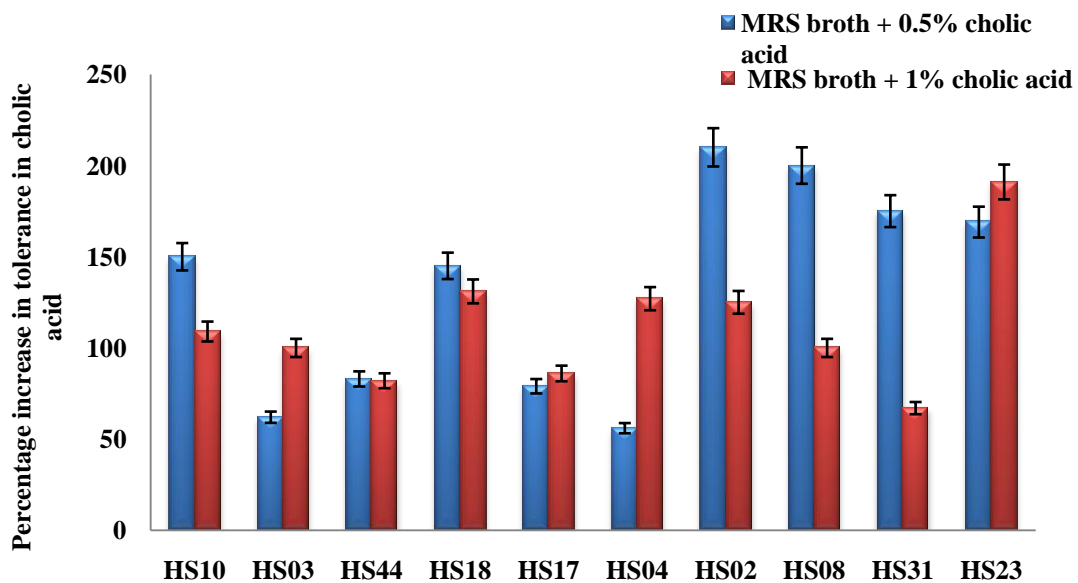
**Figure 14(c):** Percentage increase in bile tolerance by LAB in 0.5% and 1% Cholic acid after 8h.

The codes are *Enterococcus* HS1, *Enterococcus* HS03, *Enterococcus* HS44, *Enterococcus* HS18, *Lactococcus* HS17, *Enterococcus* HS04, *Enterococcus*HS02, *Enterococcus* HS08, *Enterococcus* HS31, *Enterococcus* HS23.
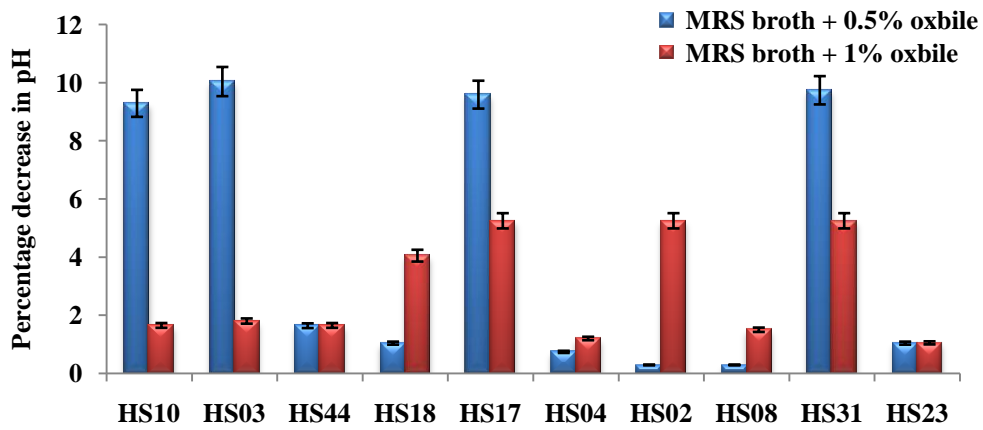
**Figure 15(a):** Percentage decrease in pH by LAB in 0.5% and 1% Ox bile
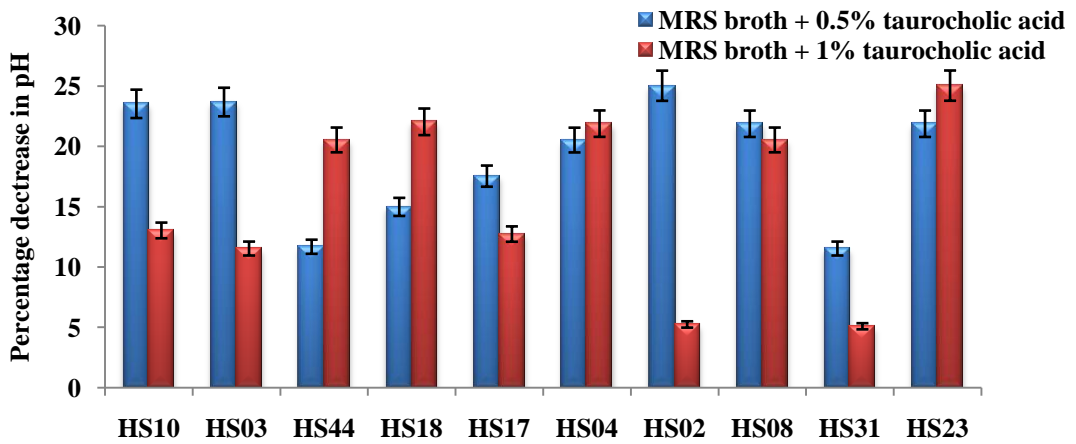


**Figure 15(b):** Percentage decrease in pH by LAB in 0.5% and 1% Taurocholic acid
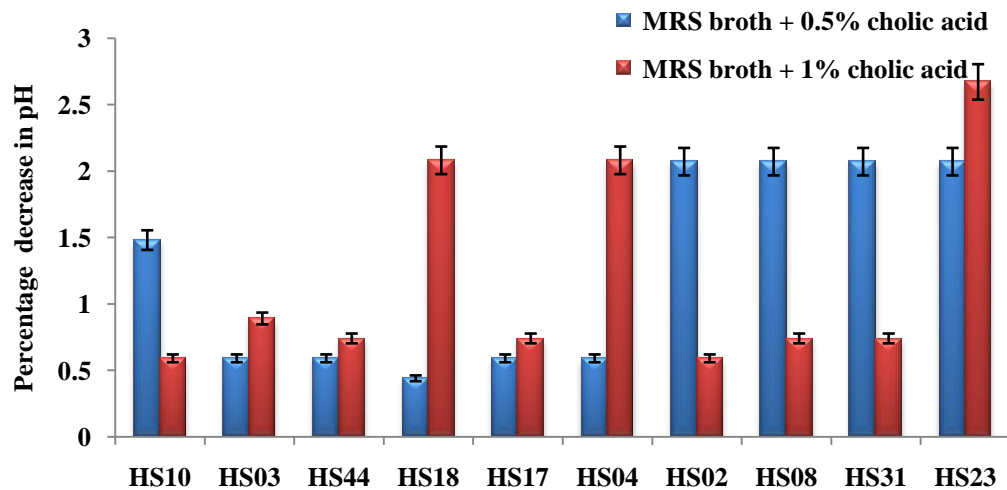


**Figure 15(c):** Percentage decrease in pH by LAB in 0.5% and 1% Cholic acid.

The codes are *Enterococcus* HS1, *Enterococcus* HS03, *Enterococcus* HS44, *Enterococcus* HS18, *Lactococcus* HS17, *Enterococcus* HS04, *Enterococcus*HS02, *Enterococcus* HS08, *Enterococcus* HS31, *Enterococcus* HS23.

## 4.7. Cell surface hydrophobicity

The cell surface hydrophobicity (CSH) values ranged from 20.50% to 94.80 in fermented Yak milk product isolates (Figure 16). The results revealed greatest hydrophobicity in n-hexadecane was observed for *Lactobacillus* YD8S followed by *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD9S and *Pediococcu*s YHC6 giving 94.80%, 75.01%, 77.68%, 79.59%, 85.18%, 71.45%, 66.94%, 62.38% respectively.
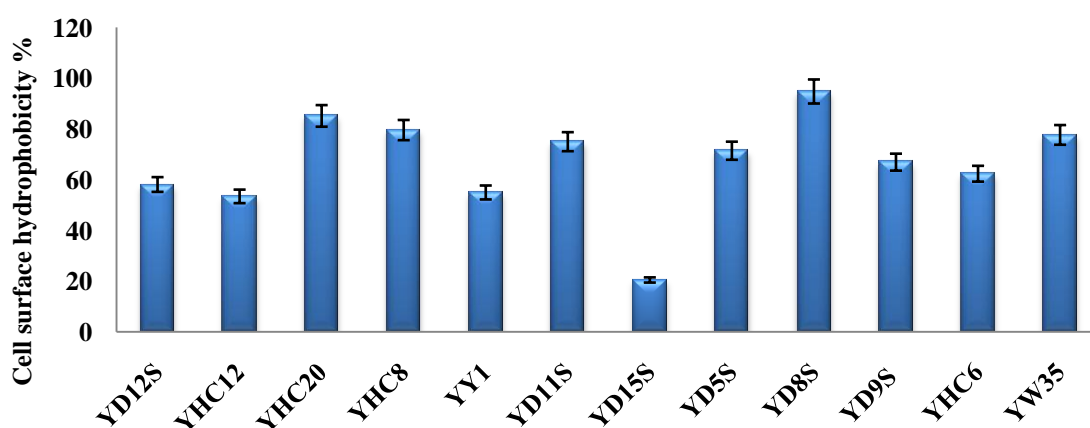


**Figure 16:** Cell Surface Hydrophobocity of the LAB (fermented Yak milk products) isolates.
Codes are *Lactobacillus* YD8S*, Enterococcus*YD12S*, Lactobacillus* YD11S*, Pediococcus* YW35*, Enterococcus* YHC8*, Enterococcus* YHC20*, Lactobacillus* YD15S*, Enterococcus* YY1*, Lactobacillus* YD5S *Lactobacillus* YD9S*, Pediococcus* YHC6*, Lactobacillus* YHC12

These isolates may be considered as a potential probiotic culture from the adherence test. In case of fecal isolates CSH ranged from as low as 6.67% to 96.61%. The highest adherence to organic solvent n- hexadecane was seen in *Enterococcus* HS23 with a hydrobhobicity percentage of 96.61% and *Enterococcus* (HS18, HS33, HS08 and HS10) showed 89.19 %, 65.07%, 49.11%, and 48.53% hydrobhobicity respectively (Figure 17). High percentage of hydrophobicity indicates the probability of isolates to adhere to the intestine of the host.
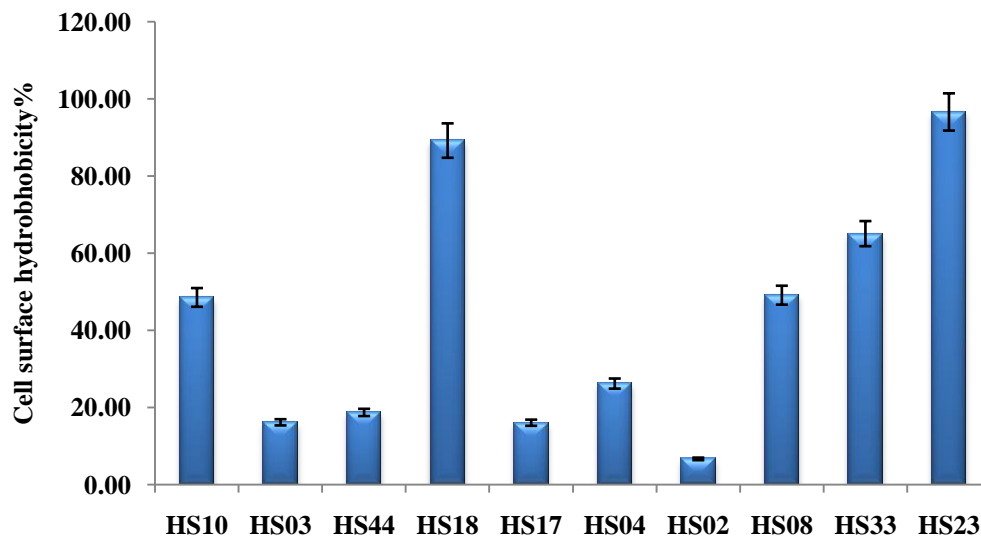
**Figure 17:** Cell Surface Hydrophobocity of the LAB (human fecal) isolates.

The codes are *Enterococcus* HS1, *Enterococcus* HS03, *Enterococcus* HS44, *Enterococcus* HS18, *Lactococcus* HS17, *Enterococcus* HS04, *Enterococcus* HS02, *Enterococcus* HS08, *Enterococcus* HS31, *Enterococcus* HS23.

## 4.8. Mechanism of cholesterol lowering by LAB

### 4.8.1. Screening for bile salt hydrolase (BSH) activity

A total of 69 isolates (35 LAB from yak milk products and 34 from human fecal sample) had been screened for BSH activity by direct plate assay. The overnight grown individual isolates were streaked in MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA) (Sigma, United States) and on MRS plates for comparison as control. The presence of opaque granular white colonies with white shine was considered as a positive reaction. Depending on how thick the precipitation was, each isolate was categorised as high denoted by "+++",

intermediate denoted by "++", low denoted by "+" and none denoted by "_" (Figure 18a, 18b and 19). The BSH activity of Fermented Yak milk product isolates have been presented in Table 16. Out of 35 isolates from yak milk product 21 were BSH positive showing dense white opaque white colonies on streaking in TDCA +MRS agar plates. From the 21 isolates only 11, *Lactobacillus* YW45, *Pediococcus* YHC21, *Enterococcus* YHC20, *Pediococcus* YHC10, *Enterococcus* YHC8, *Lactobacillus* YD9S, *Lactobacillus* YD11S, *Enterococcus* YD12S, *Lactobacillus* YD8S, *Lactobacillus* YD5S, *Enterococcus* YY1 were selected for further study on cholesterol lowering of BSH positive isolates. These 11 isolates showed maximum BSH activity.

**Table 16:** BSH activity of LAB isolates from Yak Fermented milk products

| Genera | BSH activity | Genera | BSH activity |
|---|---|---|---|
| *Enterococcus* YW1 | ++ | *Lactobacillus* YD8S | +++ |
| *Enterococcus* YY1 | +++ | *Lactobacillus* YD9S | +++ |
| *Enterococcus* YHC12 | - | *Lactobacillus* YD11S | +++ |
| *Enterococcus* YHC13 | ++ | *Lactobacillus* YD15S | - |
| *Enterococcus* YHC20 | +++ | *Lactobacillus* YC5 | - |
| *Enterococcus* YD12S | +++ | *Lactobacillus* YC5S | - |
| *Enterococcus* YHC4 | ++ | *Lactobacillus* YC6S | - |
| *Enterococcus* YHC5 | - | *Lactococcus* YCH4 | + |
| *Enterococcus* YHC8 | ++ + | *Lactococcus* YCH10 | ++ |
| *Lactobacillus* YW16 | - | *Lactococcus* YW10 | - |
| *Lactobacillus* YW41 | + | *Pediococcus* YW6 | - |
| *Lactobacillus* YW45 | +++ | *Pediococcus* YW35 | - |
| *Lactobacillus* YHC3 | + | *Pediococcus* YHC6 | - |
| *Lactobacillus* YHC11 | - | *Pediococcus* YHC7 | ++ |
| *Lactobacillus* YD3S | + | *Pediococcus* YHC9 | + |
| *Lactobacillus* YD5S | +++ | *Pediococcus* YHC10 | +++ |
| *Lactobacillus* YD6S | - | *Pediococcus* YHC21 | +++ |
| *Lactobacillus* YD7S | - | | |

High activity denoted by"+++", intermediate denoted by "++",  low denoted by "+" and none denoted by "-"

**Table 17:** BSH activity of 34 human fecal isolates

| Genera | BSH activity | Genera | BSH activity |
|---|---|---|---|
| *Enterococcus* HS01 | +++ | *Enterococcus* HS31 | +++ |
| *Enterococcus* HS02 | ++ | *Enterococcus* HS33 | +++ |
| *Enterococcus* HS03 | - | *Enterococcus* HS40 | +++ |
| *Enterococcus* HS04 | +++ | *Enterococcus* HS41 | + |
| *Enterococcus* HS05 | ++ | *Enterococcus* HS42 | ++ |
| *Enterococcus* HS06 | +++ | *Enterococcus* HS43 | +++ |
| *Enterococcus* HS08 | +++ | *Enterococcus* HS44 | - |
| *Enterococcus* HS10 | +++ | *Enterococcus* HS45 | +++ |
| *Enterococcus* HS12 | - | *Lactococcus* HS09 | ++ |
| *Enterococcus* HS13 | + | *Lactococcus* HS14 | + |
| *Enterococcus* HS18 | +++ | *Lactococcus* HS17 | +++ |
| *Enterococcus* HS21 | + | *Lactococcus* HS20 | + |
| *Enterococcus* HS23 | +++ | *Lactococcus* HS39 | ++ |
| *Enterococcus* HS24 | +++ | *Pediococcus* HS15 | **-** |
| *Enterococcus* HS25 | ++ | *Pediococcus* HS19 | +++ |
| *Enterococcus* HS26 | + | *Pediococcus* HS22 | +++ |
| *Enterococcus* HS29 | +++ | *Pediococcus* HS32 | +++ |

High activity denoted by"+++", intermediate denoted by "++", low denoted by "+" and none denoted by "-"

The screening of 34 Human faecal isolates screened by direct plate assay is presented in Table 17. Out of 34 isolates, 31 isolates showed positive results; only those isolates that showed dense precipitation were selected. Out of 18 isolates, *Enterococcus* (HS04, HS06, HS08, HS10, HS18, HS22, HS23, HS24, HS29, HS31, HS33, HS42, HS43 and HS45), *Lactococcus* HS17 and *Pediococcus* (HS32, HS19) showed maximum precipitation of bile TDCA.

There were 14 isolates from fermented yak milk products and 4 isolates from healthy human faeces that were cholesterol lowering isolates but did not show any bile salt precipitation, thereby showing no presence of bile salt hydrolase enzyme.
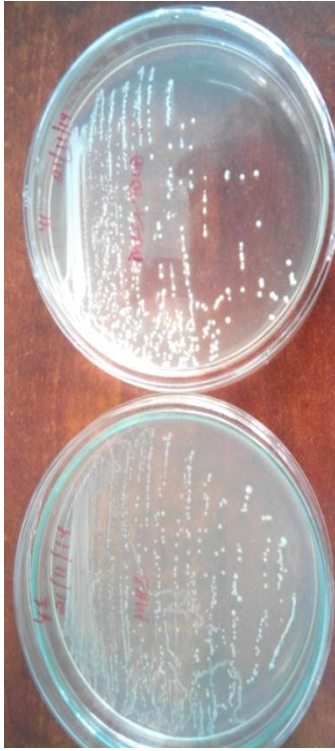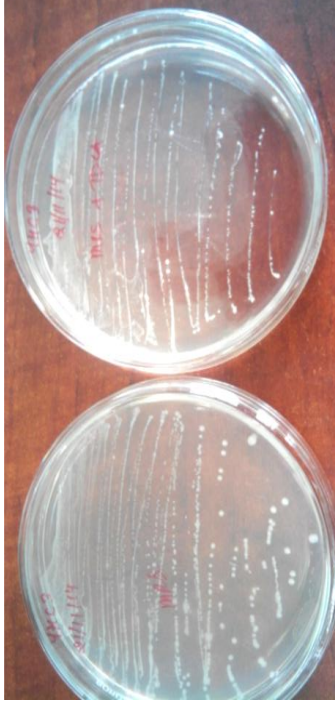
*Lactobacillus* **YHC3**
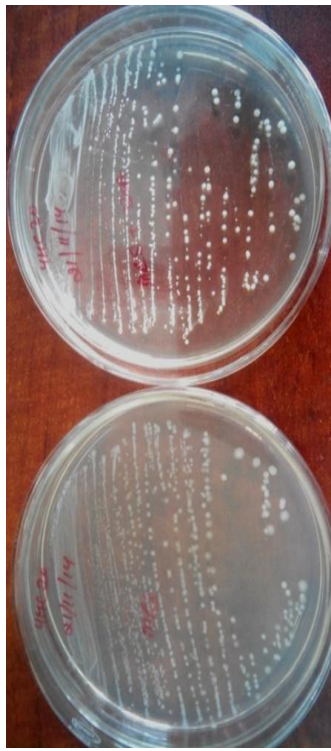
*Lactobacillus* **YW45**
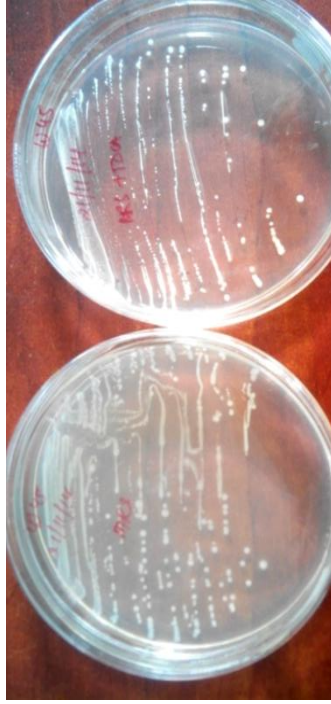
*Enterococcus* **YY1**

*Enterococcus* **YHC20**

**Figure 18a:** Detection of BSH activity of some LAB isolates of fermented yak milk product by direct plate assay. The plate on the left side contains MRS only and the plate on right side contains MRS+TDCA.

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*Lactobacillus* **YD8S**
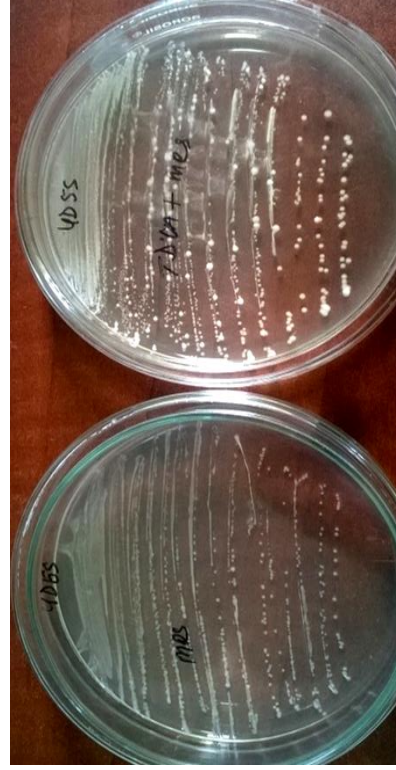
*Enterococcus* **YHC8**

*Lactobacillus* **YD5S**

**Figure 18 b:** Detection of BSH activity of LAB isolates some fermented yak milk product by direct plate assay. The plate on the left side contains MRS only and the plate on right side contains MRS+TDCA.
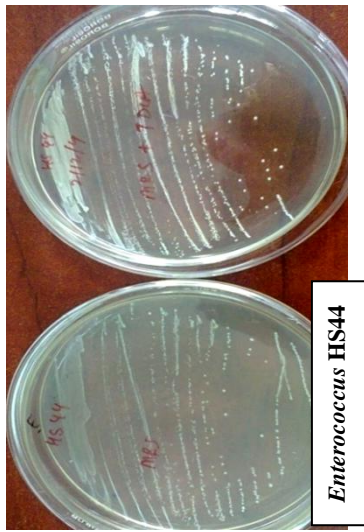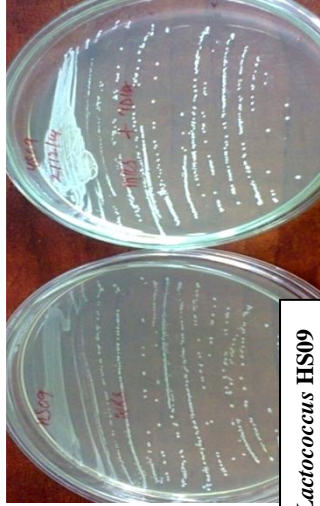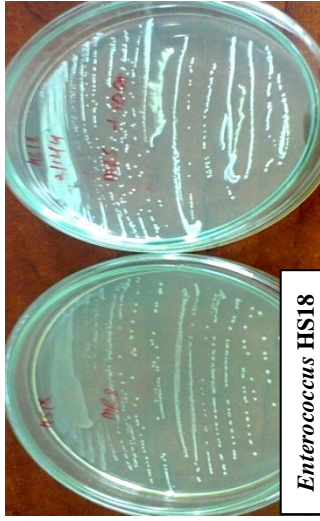
**Figure19:** Detection of BSH activity of LAB isolates of human fecal sample by direct plate assay. The plate on the left side contains MRS only and the plate on right side contains MRS + TDCA

The selected 11 isolates of fermented yak milk products and 18 isolates healthy human faeces were grouped as Group I (11 + 18) which showed BSH positive activity and the remaining isolates (14 + 4) that showed negative BSH activity was grouped into Group II.

### 4.8.2. Quantitative estimation of BSH activity

The BSH activity of LAB isolates from the fermented yak milk products have been shown in Table 18. All isolates have shown varying degrees of BSH activities towards both sodium glycocholate and sodium taurocholate (Figure 20 and Figure 21). Most of the isolates showed substrate preference towards SG compared to ST with exception to *Enterococcus* YHC8, *Lactobacillus* YD9S, *Lactococcus* YW45, *Pediococcus* YHC10 *and Enterococcus* YHC8 showing more preferences to ST. The BSH activity ranged from 0.66 to 1.30 U/ml in case of SG and 0.66 to 1.11 in case of ST in glycine and taurine conjugated bile salts. *Enterococcus* YY1 showed highest BSH activity of 1.30 U/ml in glycine conjugated bile and lowest BSH activity of 0.66 U/ml was *Pediococcus* YHC21 and *Pediococcus* YHC10 respectively. In case of Taurine conjugated bile salt, the highest BSH activity was shown by *Lactobacillus* YW45 and *Enterococcus* YHC8 as 1.11 U/ml. The lowest BSH activity was exhibited by *Pediococcus* YHC21 as 0.60 U/ml. There were varying protein content in the LAB isolates. *Enterococcus* YY1 that showed highest BSH activity also showed highest specific activity as well and lowest specific activity was shown by *Pediococcus* YHC21 in case of glycine conjugated bile salt. However the specific activity did not relate with BSH activity in *Enterococcus* YHC20, *Pediococcus* YHC10, *Enterococcus* YHC8 and *Lactobacillus* YD9S. The highest specific activity was shown by YD9S *Lactobacillus* and lowest specific activity was shown by YHC21 *Pediococcus*.

Similarly, the BSH activity of LAB isolates from healthy human faecal source have been presented in Table 19. The isolates showed substrate preference more towards SG than ST (Figure 22 and 23). Highest BSH activity was shown by *Enterococcus* HS10 as 1.03U/ml and lowest was exhibited by *Enterococcus* HS24, *Enterococcus* HS32 and *Enterococcus* HS17 as 0.66U/ml in case of glycine conjugated bile salt. The highest specific activity was shown by *Enterococcus* HS10 as 0.83 U/ mg and lowest was shown by *Pediococcus* HS32 and *Pediococcus* HS33 as 0.55 U/mg. In ST, the highest BSH activity was shown by *Enterococcus* HS10 as 0.93U/ml and lowest was shown by *Enterococcus* HS43 as 0.47 U/ml. The highest specific activity was shown by *Enterococcus* HS10 as 0.78U/mg and lowest by *Enterococcus* HS43 as 0.40U/mg. Those isolates that show ≥ 0.75U/ml BSH activity was selected for further study, there were 9 isolates from fermented yak milk samples and 8 among human faecal isolates.

### 4.8.3. Deconjugation of sodium glycocholate and sodium taurocholate

Bile salt deconjugation ability by LAB isolates from fermented yak milk products is shown in Table 20. The bile acid deconjugation was determined by the amount of cholic acid released which ranged from 0.34 to 3.30mM. All isolates were able to deconjugate both Sodium glycocholate and sodium taurocholate at different degrees. *Lactobacillus* YD11S showed better deconjugation ability as compared to other isolates. In broth containing sodium glycocholate highest deconjugation was shown by *Lactobacillus* YD11S followed by *Lactobacillus* YD5S, *Lactobacillus* YD9S, *Enterococcus* YY1, *Enterococcus* YHC20, *Enterococcus* YD12S, *Enterococcus* YHC8 and *Lactobacillus* YD8S. Lowest deconjugation of sodium glycocholate was shown by YW45 *Lactobacillus*. In case of deconjugation in sodium taurocholate containing broth highest deconjugating ability was shown by *Lactobacillus* YD11S

**Table 18:** BSH activity of LAB isolates fermented yak milk product using sodium glycocholate and sodium taurocholate as substrate

| Genera | BSH Activity | | | | | |
|---|---|---|---|---|---|---|
| | Sodium glycocholate | | | Sodium taurocholate | | |
| | Total protein mg/ml | Total activity U/ml | Specific activity U/mg | Total protein mg/ml | Total activity U/ml | Specific activity U/mg |
| *Enterococcus* YY1 | 1.21±0.11 | 1.30±0.10 | 1.07±0.11 | 1.22±0.18 | 0.93±0.11 | 0.76±0.23 |
| *Enterococcus* YHC8 | 1.21±0.56 | 0.80±0.45 | 0.66±0.11 | 1.22 ±0.21 | 1.11±0.23 | 0.90±0.34 |
| *Enterococcus* YHC20 | 1.21±0.56 | 0.92±0.32 | 0.76±0.25 | 1.24±0.19 | 0.83±0.19 | 0.67±0.15 |
| *Enterococcus* YD12S | 1.21±0.45 | 0.97±0.11 | 0.80±0.23 | 1.23±0.23 | 0.83±0.15 | 0.67±0.17 |
| *Lactobacillus* YW45 | 1.20 ±0.23 | 1.03±0.56 | 0.86±0.12 | 1.20±0.11 | 1.11±0.32 | 0.93±0.45 |
| *Lactobacillus* YD5S | 1.20±0.23 | 0.82±0.13 | 0.68±0.21 | 1.20±0.11 | 0.80±0.17 | 0.78±0.24 |
| *Lactobacillus* YD8S | 1.19±0.21 | 0.82±0.54 | 0.69±0.25 | 1.20±0.16 | 0.74±0.11 | 0.62±0.23 |
| *Lactobacillus* YD9S | 1.20±0.65 | 0.87±0.23 | 0.73±0.34 | 1.17±0.35 | 1.06±0.39 | 0.91±0.16 |
| *Lactobacillus* YD11S | 1.24±0.23 | 1.03±0.15 | 0.83±0.12 | 1.21±0.11 | 0.88±0.23 | 0.73±0.34 |
| *Pediococcus* YHC10 | 1.20±0.12 | 0.66±0.11 | 0.55±0.45 | 1.18±0.79 | 0.70±0.12 | 0.59±0.11 |
| *Pediococcus* YHC21 | 1.14±0.13 | 0.66±0.23 | 0.58±0.34 | 1.20±0.45 | 0.60±0.30 | 0.50±0.34 |

Values are means of triplicates from two separate runs, n = 2. BSH activity from cell free extracts of LAB isolates grown on MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate; ± standard error
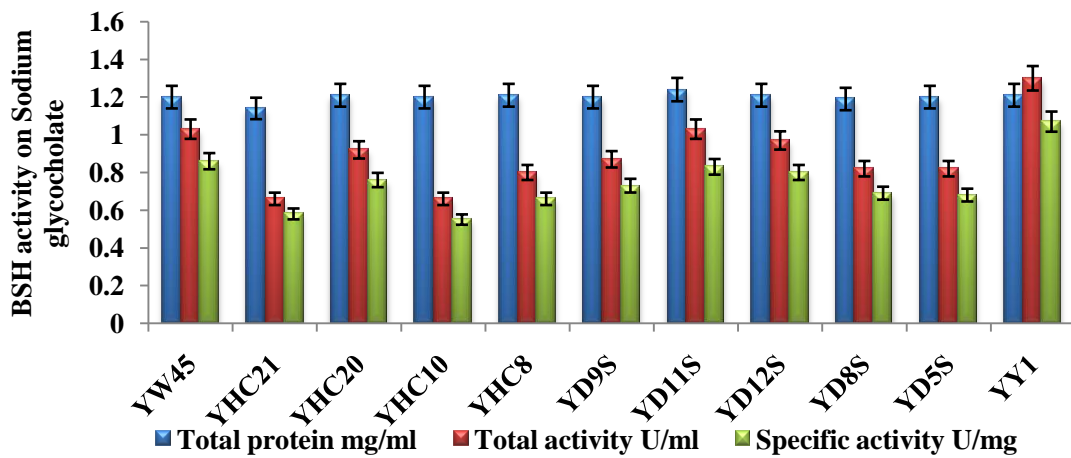
**Figure 20**: BSH activity of fermented yak milk product LAB isolates on sodium glychocholate

Codes are *Lactobacillus* YD8S, *Enterococcus*YD12S, *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD15S, *Enterococcus* YY1, *Lactobacillus* YD5S *Lactobacillus* YD9S, *Pediococcus* YHC6, *Lactobacillus* YHC12
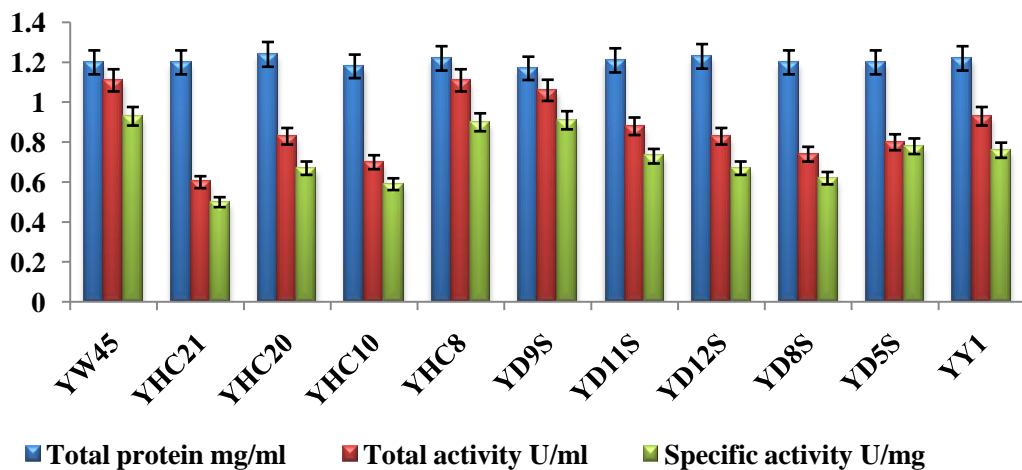


**Figure 21:** BSH activity of fermented yak milk product LAB isolates on sodium taurocholate

Codes are *Lactobacillus* YD8S, *Enterococcus*YD12S, *Lactobacillus* YD11S, *Pediococcus* YW35, *Enterococcus* YHC8, *Enterococcus* YHC20, *Lactobacillus* YD15S, *Enterococcus* YY1, *Lactobacillus* YD5S *Lactobacillus* YD9S, *Pediococcus* YHC6, *Lactobacillus* YHC12

**Table 19:** BSH activity of LAB isolates from human fecal samples using sodium glycocholate and sodium taurocholate as substrate

| | BSH Activity | | | | | |
| | Sodium glychocholate | | | Sodium taurocholate | | |
| Genera | Total protein mg/ml | Total activity U/ml | Specific activity U/mg | Total protein mg/ml | Total activity U/ml | Specific activity U/mg |
|---|---|---|---|---|---|---|
| *Enterococcus* HS01 | 1.17±0.11 | 0.67±0.29 | 0.57±0.35 | 1.17±0.16 | 0.59±0.14 | 0.50±0.27 |
| *Enterococcus* HS04 | 1.19±0.12 | 0.87±0.23 | 0.73±0.21 | 1.17±0.34 | 0.79±0.11 | 0.68±0.12 |
| *Enterococcus* HS06 | 1.17±0.23 | 0.66±0.17 | 0.56±0.23 | 1.17±0.12 | 0.60±0.54 | 0.51±0.18 |
| *Enterococcus* HS08 | 1.17±0.13 | 0.66±0.34 | 0.56±0.30 | 1.13±0.23 | 0.64±0.26 | 0.57±0.28 |
| *Enterococcus* HS10 | 1.24±0.65 | 1.03±0.12 | 0.83±0.23 | 1.20±0.58 | 0.93±0.19 | 0.78±0.21 |
| *Enterococcus* HS18 | 1.17±0.11 | 0.82±0.54 | 0.70±0.34 | 1.15±0.23 | 0.79±0.12 | 0.68±0.12 |
| *Enterococcus* HS23 | 1.17±0.54 | 0.82±0.56 | 0.70±0.12 | 1.17±0.34 | 0.77±0.23 | 0.66±0.24 |
| *Enterococcus* HS24 | 1.17±0.19 | 0.66±0.12 | 0.56±0.67 | 1.17±0.50 | 0.60±0.56 | 0.51±0.23 |
| *Enterococcus* HS29 | 1.17±0.11 | 0.82±0.17 | 0.70±0.15 | 1.17±0.19 | 0.70±0.18 | 0.60±0.12 |
| *Enterococcus* HS31 | 1.20±0.34 | 1.03±0.11 | 0.86±0.64 | 1.20±0.13 | 0.93±0.22 | 0.78±0.45 |

**Table 19 continued:** BSH activity of human faecal LAB isolates on sodium glycocholate and sodium taurocholate as substrate

<div align="center">BSH Activity</div>

| Genera | Sodium glychocholate | | | Sodium taurocholate | | |
|---|---|---|---|---|---|---|
| | Total protein mg/ml | Total activity U/ml | Specific activity U/mg | Total protein mg/ml | Total activity U/ml | Specific activity U/mg |
| *Enterococcus* HS33 | 1.20±0.23 | 0.66±0.34 | 0.55±0.45 | 1.17±0.23 | 0.64±0.65 | 0.54±0.45 |
| *Enterococcus* HS40 | 1.20±0.12 | 0.82±0.34 | 0.68±0.90 | 1.17±0.12 | 0.79±0.32 | 0.68±0.23 |
| *Enterococcus* HS43 | 1.17±0.15 | 0.66±0.23 | 0.56±0.34 | 1.18±0.35 | 0.47±0.15 | 0.40±0.12 |
| *Enterococcus* HS45 | 1.24±0.17 | 1.03±0.23 | 0.84±0.34 | 1.20±0.40 | 0.79±0.19 | 0.66±0.54 |
| *Lactococcus* HS17 | 1.16±0.12 | 0.66±0.44 | 0.57±0.29 | 1.17±0.13 | 0.60±0.39 | 0.51±0.30 |
| *Pediococcus* HS19 | 1.25±0.24 | 0.66±0.18 | 0.53±0.11 | 1.20±0.45 | 0.60±0.12 | 0.50±0.27 |
| *Pediococcus* HS22 | 1.16±0.20 | 0.66±0.32 | 0.57±0.36 | 1.17±0.49 | 0.60±0.23 | 0.51±0.21 |
| *Pediococcus* HS32 | 1.20±0.13 | 0.66±0.23 | 0.55±0.56 | 1.17±0.45 | 0.64±0.23 | 0.54±0.45 |

Values are means of triplicates from two separate runs, n = 2. BSH activity from cell free extracts of LAB isolates grown on MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate.± standard error
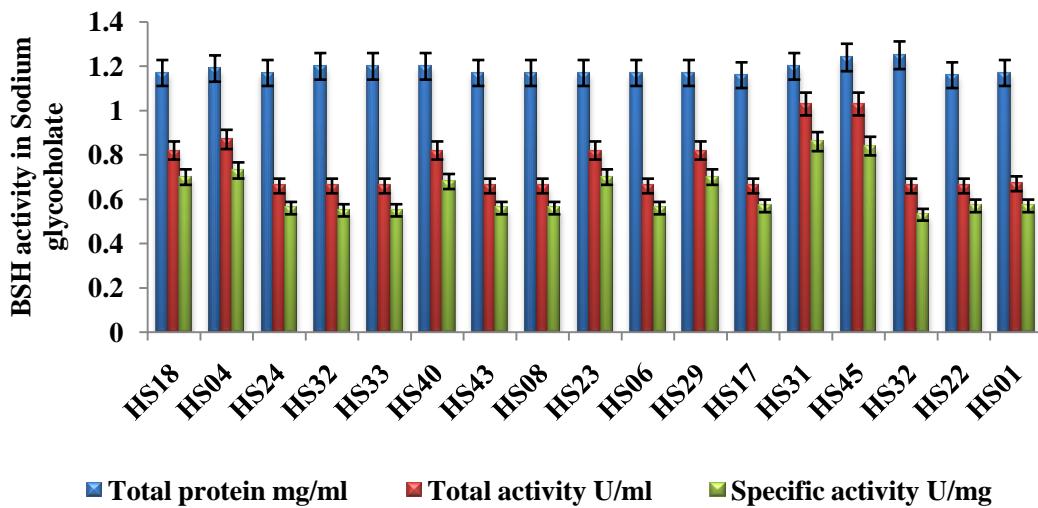
**Figure 22:** BSH activity of LAB isolates from human faecal on sodium glycocholate

Codes are *Enterococcus* HS18, *Enterococcus* HS04, *Enterococcus*HS24, *Pediococcus* HS32, *Enterococcus*HS33, *Enterococcus* HS40, *Enterococcus* HS43, *Enterococcus* HS08. *Enterococcus*HS23, *Enterococcus* HS06, *Enterococcus* HS29, *Lactococcus* HS17, *Enterococcus*HS31, *Enterococcus*HS45, *Pediococcus* HS32, *Pediococcus* HS22, *Enterococcus*HS01, *Enterococcus*HS10.
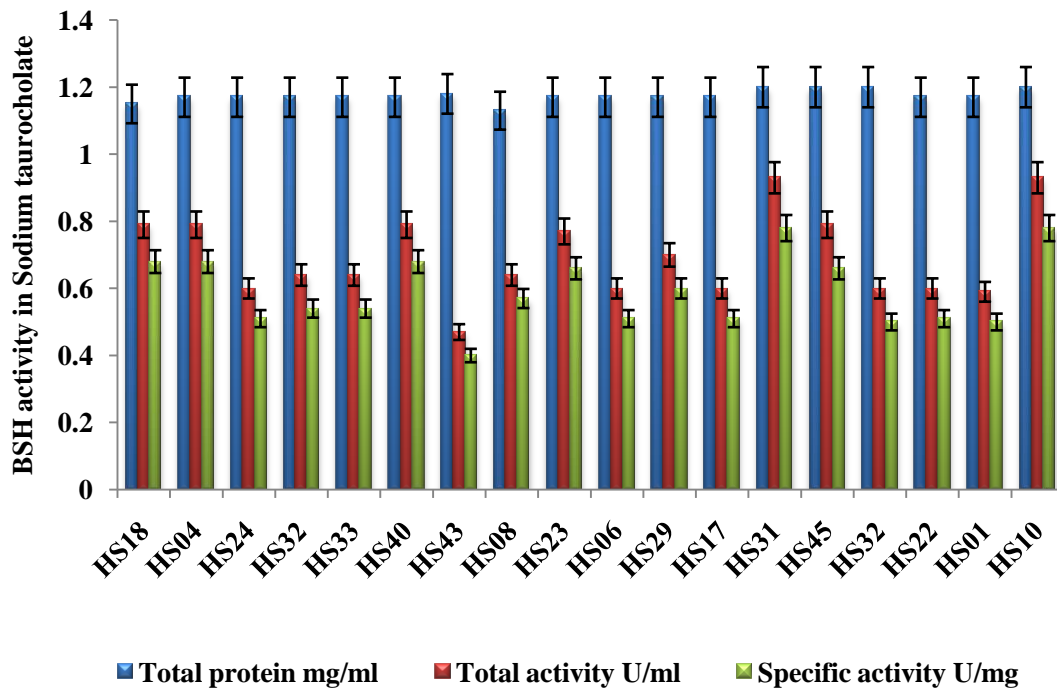


**Figure 23:** BSH activity of LAB isolates from human faecal on sodium taurocholate

Codes are *Enterococcus* HS18, *Enterococcus* HS04, *Enterococcus*HS24, *Pediococcus* HS32, *Enterococcus*HS33, *Enterococcus* HS40, *Enterococcus* HS43, *Enterococcus* HS08. *Enterococcus*HS23, *Enterococcus* HS06, *Enterococcus* HS29, *Lactococcus* HS17, *Enterococcus*HS31, *Enterococcus*HS45, *Pediococcus* HS32, *Pediococcus* HS22, *Enterococcus*HS01, *Enterococcus*HS10.

**Table 20:** Bile salt deconjugation by LAB  from fermented milk product

| Genera | Cholic acid released (mM) | |
|---|---|---|
| | Sodium Glycocholate | Sodium Taurocholate |
| *Enterococcus* YY1 | 1.79±0.10 | 0.66±0.26 |
| *Enterococcus* YD12S | 1.62±0.13 | 0.40±0.24 |
| *Enterococcus* YHC8 | 1.40±0.34 | 0.49±0.31 |
| *Enterococcus* YHC20 | 1.67±0.11 | 1.64±0.12 |
| *Lactobacillus* YD5S | 2.47±0.11 | 0.49±0.10 |
| *Lactobacillus* YD8S | 1.40±0.26 | 1.25±0.28 |
| *Lactobacillus* YD9S | 1.97±0.24 | 1.96±0.21 |
| *Lactobacillus* YD11S | 3.30±0.35 | 1.98±0.50 |
| *Lactobacillus* YW45 | 1.10±0.23 | 0.34±11 |

**Table 21:** Bile salt deconjugation by LAB from healthy human fecal sample

| Genera | Cholic acid released (mM) | |
|---|---|---|
| | Sodium Glycocholate | Sodium Taurocholate |
| *Enterococcus* HS04 | 1.47±0.34 | 0.98±0.54 |
| *Enterococcus* HS10 | 0.83±0.43 | 1.47±0.37 |
| *Enterococcus* HS18 | 1.64±0.13 | 1.27±0.15 |
| *Enterococcus* HS23 | 0.83±0.27 | 0.60±0.46 |
| *Enterococcus* HS29 | 1.81±0.25 | 0.83±0.30 |
| *Enterococcus* HS31 | 0.83±0.23 | 0.66±0.27 |
| *Enterococcus* HS40 | 1.81±0.23 | 1.32±0.25 |
| *Enterococcus* HS45 | 0.98±0.18 | 0.98±0.25 |

Values are means of triplicates from two separate runs, n = 2. Deconjugation of glycine ortaurine conjugated bile based on release of cholic acid. MRS broth supplemented with 6 mMsodium glycocholate; 6 mM sodium taurocholate

followed by *Lactobacillus* YD9S, *Enterococcus* YY1, *Enterococcus* YHC20 and *Lactobacillus* YD8S, the lowest deconjugation was shown by *Lactobacillus* YW45**.** The isolates showed lower deconjugation in sodium taurocholate than sodium glycocholate. The bile acid deconjugation of LAB isolates from healthy human faecal samples is presented in Table 21. The isolates showed varying degrees of deconjugation in broth containing sodium glycocholate and Sodium taurocholate. However, deconjugation was found to be more in sodium glycocholate than sodium

taurocholate. The deconjugation ability in sodium glycocholate ranged from 0.83 to 1.8mM. The highest activity was shown by *Enterococcus* HS29 and lowest by *Enterococcus* HS23, *Enterococcus* HS10 and *Enterococcus* HS31 being 0.83mM. In sodium taurocholate the highest deconjugation ability is observed in *Enterococcus* HS10 as 1.47mM and lowest in *Enterococcus* HS23 as 0.60 mM.

### 4.8.4. Co-precipitation of bile salts by LAB

Co-precipation of cholesterol with cholic acid is liberated from the deconjugation of sodium glycocholate and sodium taurocholate by LAB isolates from fermented Yak milk product is shown in Table 22. Cholesterol was co-precipitated with deconjugation of both sodium glycocholate and sodium taurocholate at varying degrees. Precipitation of cholesterol upon deconjugation of sodium glycocholate ranged from 1.4 to 5.6 μg/ml which was higher compared to sodium taurocholate which ranged from 0.2 to 2.3 μg/ml. Highest cholesterol precipitation in SG containing broth was shown by *Enterococcus* YD12S, *Lactobacillus* YD9S, *Enterococcus* YY1, and *Lactobacillus* YD8S were ≥3.2 μg/ml and lowest co-precipitation with deconjugation was shown by *Lactobacillus* YW45 and *Enterococcus* YHC8 as 1.4 μg/ml. Cholesterol precipitation with sodium taurocholate by *Enterococcus* YY1 was higher compared to other isolates. Highest cholesterol precipitation was obtained from *Enterococcus* YY1 followed by *Lactobacillus* YD5S,

*Lactobacillus* YD8S, *Enterococcus* YHC20 and *Lactobacillus* YD11S with *Lactobacillus* YW45 showing the lowest cholesterol precipitation. In human faecal isolates the cholesterol precipitation with sodium glycocholate ranged from 0.9 to 5μg/ml and with sodium taurocholate the cholesterol precipitation ranged from 0.2 to 1.1μg/ml that has been shown in Table 23. The highest cholesterol precipitation with

**Table 22:** Cholesterol precipitation by LAB from fermented milk product in presence of sodium glycocholate and sodium taurocholate

| Genera | Cholesterol precipitated (µg/ml) | |
| --- | --- | --- |
| | Sodium Glycocholate | Sodium Taurocholate |
| *Enterococcus* YY1 | 4.0±0.11 | 2.3±0.16 |
| *Enterococcus* YD12S | 5.6±0.24 | 1.2±0.23 |
| *Enterococcus* YHC8 | 1.4±0.10 | 1.5±0.23 |
| *Enterococcus* YHC20 | 2.3±0.12 | 1.8±0.23 |
| *Lactobacillus* YD9S | 4.0±0.34 | 1.4±0.12 |
| *Lactobacillus* YD5S | 2.9±0.15 | 2.0±0.17 |
| *Lactobacillus* YD8S | 3.2±0.15 | 2.1±0.21 |
| *Lactobacillus* YD11S | 1.9±0.23 | 1.7±0.34 |
| *Lactobacillus* YW45 | 1.4±0. 23 | 0.2±0.11 |

**Table 23:** Cholesterol precipitation by LAB from  human fecal  sample in presence of sodium glycocholate and sodium taurocholate

| Genera | Cholesterol precipitated (µg/ml) | |
| --- | --- | --- |
| | Sodium Glycocholate | Sodium Taurocholate |
| *Enterococcus* HS04 | 5.0±0.17 | 1.1±0.10 |
| *Enterococcus* HS10 | 0.9±0.29 | 0.3±0.11 |
| *Enterococcus* HS18 | 1.9±0.12 | 1.0±0.24 |
| *Enterococcus* HS23 | 1.7±0.16 | 1.0±0.11 |
| *Enterococcus* HS29 | 3.3±0.17 | 1.0±0.12 |
| *Enterococcus* HS31 | 1.2±0.15 | 0.2±0.17 |
| *Enterococcus* HS40 | 1.4±0.36 | 0.2±0.23 |
| *Enterococcus* HS45 | 1.1±0.25 | 0.7±0.11 |

Values are means of triplicates from two separate runs, n = 2. MRS broth supplemented with cholesterol and 6 mM sodium glycocholate; 6 mM sodium taurocholate

sodium glycocholate was shown by *Enterococcus* HS04 and lowest by *Enterococcus* HS10. Similarly the cholesterol precipitation with sodium taurocholate, the highest was shown by *Enterococcus* HS04 as 1.1µg/ml and lowest by *Enterococcus* HS40 and *Enterococcus* HS31 as 0.2 µg/ml.

### 4.8.5. Incorporation of cholesterol into the cellular membrane

The cholesterol incorporation in the cellular membrane of the LAB isolates has been presented in Table 24. The cholesterol incorporation by LAB isolates from two different sources has shown varying percentages depending on the isolate type. The cholesterol incorporation of 18 isolates ranged from 10 to 72%. The highest degree of incorporation was observed in case of *Enterococcus* YHC12 with the percentage of 72%, followed by *Enterococcus* HS03 (70%), *Lactobacillus* YD15S (68%), *Enterococcus* HS44 (64%) and *Enterococcus* HS12 (62%). The lowest cholesterol incorporation was shown by the isolate *Lactobacillus* YD6S being 10%.

### 4.8.6. Assimilation of cholesterol in presence of carbohydrates and lipid in media

Group II isolates include those which are BSH negative ones but cholesterol lowering; there were 11 Yak milk product isolates and 4 human faecal isolates. The cholesterol assimilation during 24 h of growth of 18 LAB isolates is presented in Table 25. Cholesterol lowering or removal varied from isolate to isolate. The values of cholesterol assimilation percentage ranged from 21% to 93% in MRS and cholesterol broth and 17% to 90% in MRS, cholesterol and 0.3% Ox gall broth. However the assimilation was more in MRS and cholesterol broth compared to MRS, cholesterol and 0.3% Ox gall broth. The highest cholesterol assimilation was shown by *Enterococcus* YHC12 with 93% in MRS and cholesterol broth and 90% in MRS,

**Table 24:** Cholesterol incorporation by the Group II LAB isolates

| Source | Genera | Cholesterol incorporation% |
|---|---|---|
| Fermented Yak milk products | *Enterococcus* YHC5 | 34±0.60 |
| | *Enterococcus* YHC12 | 72±1.74 |
| | *Lactobacillus* YW16 | 20±1.75 |
| | *Lactobacillus* YC5 | 30±1.75 |
| | *Lactobacillus* YC5S | 20±0.20 |
| | *Lactobacillus* YC6S | 36±1.73 |
| | *Lactobacillus* YHC11 | 20±0.45 |
| | *Lactobacillus* YD6S | 10±1.8 |
| | *Lactobacillus* YD7S | 19±0.80 |
| | *Lactobacillus* YD15S | 68±2.6 |
| | *Lactococcus* YW10 | 25±2.30 |
| | *Pediococcus* YW6 | 30±0.50 |
| | *Pediococcus* YW35 | 43±1.78 |
| | *Pediococcus* YHC6 | 30±1.73 |
| Human fecal | *Enterococcus* HS03 | 70±1.73 |
| | *Enterococcus* HS12 | 62±2.80 |
| | *Enterococcus* HS44 | 64±1.76 |
| | *Pediococcus* HS15 | 23±1.35 |

Values are means of triplicates from two separate runs, n = 2

cholesterol and 0.3% Ox gall broth. *Lactobacillus* YD15S showed 73% and 67%.

Among the four human fecal isolates the highest cholesterol assimilation was shown

by *Enterococcus* HS03 as 85% in MRS and cholesterol broth and 79% in MRS,

cholesterol and 0.3% Ox gall broth. The lowest cholesterol assimilation percentage

shown by *Lactococcus* YW10 in MRS and cholesterol broth as 21% and

*Lactobacillus* YHC11 in MRS, cholesterol and 0.3% Ox gall broth as 17%.

## 4.9. Identification of LAB isolates by phenotypic method

The 35 LAB isolates from the fermented Yak milk samples and 34 isolates from the

healthy human fecal origin had been identified up to genus level of LAB. These

**Table 25:** Cholesterol assimilation of the Group II LAB isolates

| Source | Genera | Cholesterol assimilation % | |
| --- | --- | --- | --- |
| | | MRS broth +cholesterol | MRS broth +cholesterol+ 0.3% Ox gal |
| Fermented Yak milk Products sample | *Enterococcus* YHC5 | 65±2.80 | 60±0.50 |
| | *Enterococcus* YHC12 | 93±0.50 | 90±0.50 |
| | *Lactobacillus* YW16 | 25±0.80 | 19±2.45 |
| | *Lactobacillus* YC5 | 35±0.50 | 31±0.50 |
| | *Lactobacillus* YC5S | 56±2.3 | 50±1.74 |
| | *Lactobacillus* YC6S | 36±2.90 | 30±1.43 |
| | *Lactobacillus* YHC11 | 29±0.50 | 17±1.75 |
| | *Lactobacillus* YD6S | 22±1.10 | 18±0.50 |
| | *Lactobacillus* YD7S | 25±1.07 | 21±0.50 |
| | *Lactobacillus* YD15S | 73±0.50 | 67±±1.89 |
| | *Lactococcus* YW10 | 21±0.50 | 20±0.50 |
| | *Pediococcus* YW6 | 30±0.50 | 25±0.50 |
| | *Pediococcus* YW35 | 67±1.73 | 64±1.73 |
| | *Pediococcus* YHC6 | 39±1.34 | 35±1.73 |
| Human fecal sample | *Enterococcus* HS03 | 85±2.5 | 79±1.78 |
| | *Enterococcus* HS12 | 48±0.50 | 41±1.73 |
| | *Pediococcus* HS15 | 45±1.01 | 31±1.56 |
| | *Enterococcus* HS44 | 69±0.98 | 64±1.73 |

Values are means of triplicates from two separate runs, n = 2.

isolates were the ones showing cholesterol lowering and probiotc property. Depending on the studies on the mechanism of cholesterol lowering the isolates had been divided under two groups: Group I containing Bile salt hydrolase positive isolates that lower cholesterol because of the presence of BSH genes. The presence of BSH genes enables deconjugation of the bile salts thereby preventing the free bile to get adsorbed to the small intestine disrupting the cholesterol absorption by the liver cells. The first group consists of 29 LAB isolates (11 Fermented Yak milk LAB and 18 Healthy human faecal LAB), out of which 6 Fermented Yak milk LAB isolates (*Lactobacillus* YD8S, *Lactobacillus* YD5S, *Lactobacillus* YD9S, *Lactobacillus*

YD11S, *Enterococcus* YHC20 and *Enterococcus* YY1) and 5 Healthy human faecal LAB isolates (*Enterococcus* HS18, *Enterococcus* HS10, *Enterococcus* HS23, *Enterococcus* HS31, *Enterococcus* HS04) were selected for identification by 16srRNA sequencing. Group II isolates contain BSH negative isolates that lower cholesterol by cellular incorporation. There were 18 LAB isolates (14 Fermented Yak milk LAB and 4 Healthy human faecal LAB), from which 3 isolates, 1 Fermented Yak milk LAB isolate (*Enterococcus* YHC12) and 2 Healthy human faecal LAB isolates (*Enterococcus* HS03 and *Enterococcus* HS44) were selected keeping in mind the percentage of cholesterol assimilation, cellular incorporation percentage and its additional probiotic properties. Sugar fermentation tests of the selected and 14 identified strains were done using the carbohydrate fermentation kit, Hi Carbo$^{TM}$ Kit (Hi Media) for further confirmation. Overnight grown cultures with an optical density of 0.5 O.D. at 620 nm were used. 50µl culture was inoculated to 35 different carbohydrates in three parts, part A and part B1 containing 12 each for carbohydrate utilization tests and part C for 11 different sugars. The plates were then incubated at 37 $^o$C for 18-24 h. After incubation the results were noted and matched with the previous available literature. The carbohydrate utilization of the identified isolates has been presented in Table 26, 27 and 28. The kit of Part A contained 12 sugars namely; lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L- arabinose and mannose. The carbohydrate utilization profile of part A kit has been presented in Table 26 where the different identified strains behave differently. *Lactobacillus* YD8S gave all test positive indicated by the change in color of the medium to yellow. *Lactobacillus* YD5S and *Lactobacillus* YD9S gave positive for all the carbohydrates except xylose which was not utilized by this strain. Similarly, *Enterococcus* YY1 gave positive test for only melibiose and L- arabinose.

*Enterococcus* HS03 gave positive result for lactose, maltose, fructose, dextrose, galactose, trehalose and mannose. *Lactobacillus* YD11S gave positive test for lactose, maltose, fructose, dextrose, galactose, trehalose, melibiose, sucrose, L-arabinose and mannose. *Enterococcus* YHC20, *Enterococcus* HS18, *Enterococcus* HS10, *Enterococcus* HS23, *Enterococcus* HS31, *Enterococcus* HS04. *Enterococcus* YHC12 and *Enterococcus* HS44 gave positive result for lactose, maltose, fructose, dextrose, galactose, trehalose, melibiose, sucrose, and mannose. The carbohydrate fermentation test using the Part B1 of the Hi Carbo $^{TM}$ Kit (Hi Media) has been presented in Table 27. Inulin, sodium gluconate, glycerol, salicin, dulicitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol and α methyl D glucosidase. The strains gave negative results for sugars like inulin, dulicitol, inositol, adonitol, erythritol and α methyl D glucosidase. *Lactobacillus* YD8S gave positive  for the utilization of salicin, sorbitol, mannitol and glycerol. Glycerol was only utilized by this strain. *Lactobacillus* YD5S and *Lactobacillus* YD9S gave positive test for the utilization of sodium gluconate, salicin, sorbitol, mannitol and arabitol. *Lactobacillus* YD11S gave positive for sodium gluconate, salicin, sorbitol, mannitol and arabitol. *Enterococcus* YY1 was able to utilize salicin and mannitol.  *Enterococcus* HS10 and *Enterococcus* HS04 showed a variation in the utilization of Sodium gluconate giving a positive on comparing with the rest of *Enterococcus* strains. However, all the strains of *Enterococcus* were able to use salicin and manitol. *Enterococcus* HS03 used only salicin from the kit B1. The part C of the kit contained rhamnose, cellobiose, melizitose, α methyl mannoside, xylitol, ONPG, esculin, D- arabinose, citrate, malonate and sorbose (Table 28). The *Lactobacillus* strains were not able to utilize xylitol, ONPG, malonate and sorbose. *Lactobacillus* YD8S gave positive result for cellobiose, melizitose, esculin and D- srabinose. *Lactobacillus* YD5S and

*Lactobacillus* YD9S utilized cellobiose, melizitose, α methyl mannoside, esculin and D- arabinose. *Lactobacillus* YD11S was able to utilize rhamnose, aellobiose, melizitose, esculin and D- arabinose. All *Enterococcus* strains gave negative results for 11 sugars except *Enterococcus* HS03 showing positive test for cellobiose.

**Table 26:** Carbohydrate fermentation test using KB009A of Hi Carbo kit (Part A)

| Isolate | Carbohydrate fermentation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lactose | Xylose | Maltose | Fructose | Dextose | Galactose | Raffinose | Trehalose | Melibiose | Sucrose | L-Arabinose | Mannose |
| *Lactobacillus* YD8S | + | + | + | + | + | + | + | + | + | + | + | + |
| *Lactobacillus* YD5S | + | - | + | + | + | + | + | + | + | + | + | + |
| *Lactobacillus* YD11S | + | - | + | + | + | + | - | + | + | + | + | + |
| *Lactobacillus* YD9S | + | - | + | + | + | + | + | + | + | + | + | + |
| *Enterococcus* YY1 | - | - | - | - | - | - | - | - | + | - | + | - |
| *Enterococcus* YHC20 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* HS18 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* HS10 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* HS23 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* HS31 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* HS04 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* YHC12 | + | - | + | + | + | + | - | + | + | + | - | + |
| *Enterococcus* HS03 | + | - | + | + | + | + | - | + | - | - | - | + |
| *Enterococcus* HS44 | + | - | + | + | + | + | - | + | + | + | - | + |

"+" indicates a positive result and "-" indicates a negative result

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**Table 27:** Carbohydrate fermentation test using KB009B1 of Hi Carbo kit (Part B1)

| Isolate | Carbohydrate fermentation | | | | |
| --- | --- | --- | --- | --- | --- |
| | Sodium Gluconate | Glycerol | Sorbitol | Mannitol | Arabitol |
| *Lactobacillus* YD8S | + | + | + | + | - |
| *Lactobacillus* YD5S | + | - | + | + | + |
| *Lactobacillus*YD11S | + | - | - | + | - |
| *Lactobacillus* YD9S | + | - | + | + | + |
| *Enterococcus* YY1 | - | - | - | + | - |
| *Enterococcus* YHC20 | - | - | - | + | - |
| *Enterococcus*  HS18 | - | + | - | + | - |
| *Enterococcus* HS10 | + | - | - | + | - |
| *Enterococcus*  HS23 | - | - | - | - | - |
| *Enterococcus*  HS31 | - | - | - | - | - |
| *Enterococcus*  HS04 | + | - | - | - | - |
| *Enterococcus* YHC12 | - | - | - | + | - |
| *Enterococcus* HS03 | - | - | - | - | - |
| *Enterococcus* HS44 | - | - | - | + | - |

All isolates showed positive test for salicin, and negative test for inulin, dulicitol, inositol, adonitol and erythritol , "+" indicates a positive result and "-" indicates a negative result

**Table 28:** Carbohydrate fermentation test using KB009C of Hi Carbo kit (Part C)

| Isolate | Carbohydrate fermentation | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | Rhamnose | Cellobiose | Melezitose | α methyl D glucosidase | Esculin | D- Arabinose | Tentative identity |
| *Lactobacillus* YD8S | - | + | + | - | + | + | *Lactobacillus pentosus* |
| *Lactobacillus* YD5S | - | + | + | + | + | + | *Lactobacillus plantarum* |
| *Lactobacillus* YD11S | + | + | + | - | + | + | *Lactobacillus paraplantarum* |
| *Lactobacillus* YD9S | - | + | + | + | + | + | *Lactobacillus plantarum* |
| *Enterococcus* YY1 | - | - | - | - | - | - | *Enterococcus lactis* |
| *Enterococcus* YHC20 | - | - | - | - | - | - | *Enterococcus faecium* |
| *Enterococcus* HS18 | - | - | - | - | - | - | *Enterococcus sp.* |
| *Enterococcus* HS10 | - | - | - | - | - | - | *Enterococcus faecium* |
| *Enterococcus* HS23 | - | - | - | - | - | - | *Enterococcus sp.* |
| *Enterococcus* HS31 | - | - | - | - | - | - | *Enterococcus sp.* |
| *Enterococcus* HS04 | - | - | - | - | - | - | *Enterococcus sp.* |
| *Enterococcus* YHC12 | - | - | - | - | - | - | *Enterococcus faecium* |
| *Enterococcus*  HS03 | - | + | - | - | - | - | *Enterococcus durans* |
| *Enterococcus* HS44 | - | - | - | - | - | - | *Enterococcus faecium* |

All isolates negative test for sorbose, malonate, citrate, ONPG and xylitol, "+" indicates a positive result and "-" indicates a negative result

**4.10. Genotypic identification by 16S rRNA sequencing**

According to 16S rRNA gene sequencing results, the LAB isolates were identified as *Lactobacillus* spp. (four strains) and *Enterococcus* spp. (ten strains). List of the strains along with their accession number are presented in the Table 29. The genus *Lactobacillus* was dominant in fermented yak milk products whereas only *Enterococcus* genus was found in the human sample. The phylogenetic tree of the yak milk product isolates with neighbor-joining statistical method (no. of bootstrap replications = 1000) is presented in Figure 24.

**Table 29:** Identity of LAB isolates and their accession numbers

| Isolate code | Identity | Accession number from NCBI | No. of base pairs |
|---|---|---|---|
| **Group I (BSH positive)** | | | |
| YD8S | *Lactobacillus pentosus* | KU601439 | 1452 |
| YD5S | *Lactobacillus plantarum* | KU601440 | 1480 |
| YD11S | *Lactobacillus paraplantarum* | KU601441 | 1472 |
| YD9S | *Lactobacillus plantarum* | KU601442 | 1012 |
| YY1 | *Enterococcus lactis* | KU601443 | 1472 |
| YHC20 | *Enterococcus faecium* | KU601444 | 769 |
| HS18 | *Enterococcus thailandicus* | KX274031 | 1407 |
| HS10 | *Enterococcus  faecium* | KX274032 | 922 |
| HS23 | *Enterococcus durans* | KX274033 | 941 |
| HS31 | *Enterococcus durans* | KX274034 | 930 |
| HS04 | *Enterococcus durans* | KX274035 | 943 |
| **Group II (BSH negative)** | | | |
| YHC12 | *Enterococcus faecium* | KX387371 | 738 |
| HS03 | *Enterococcus durans* | KX274030 | 881 |
| HS44 | *Enterococcus faecium* | KX354351 | 485 |
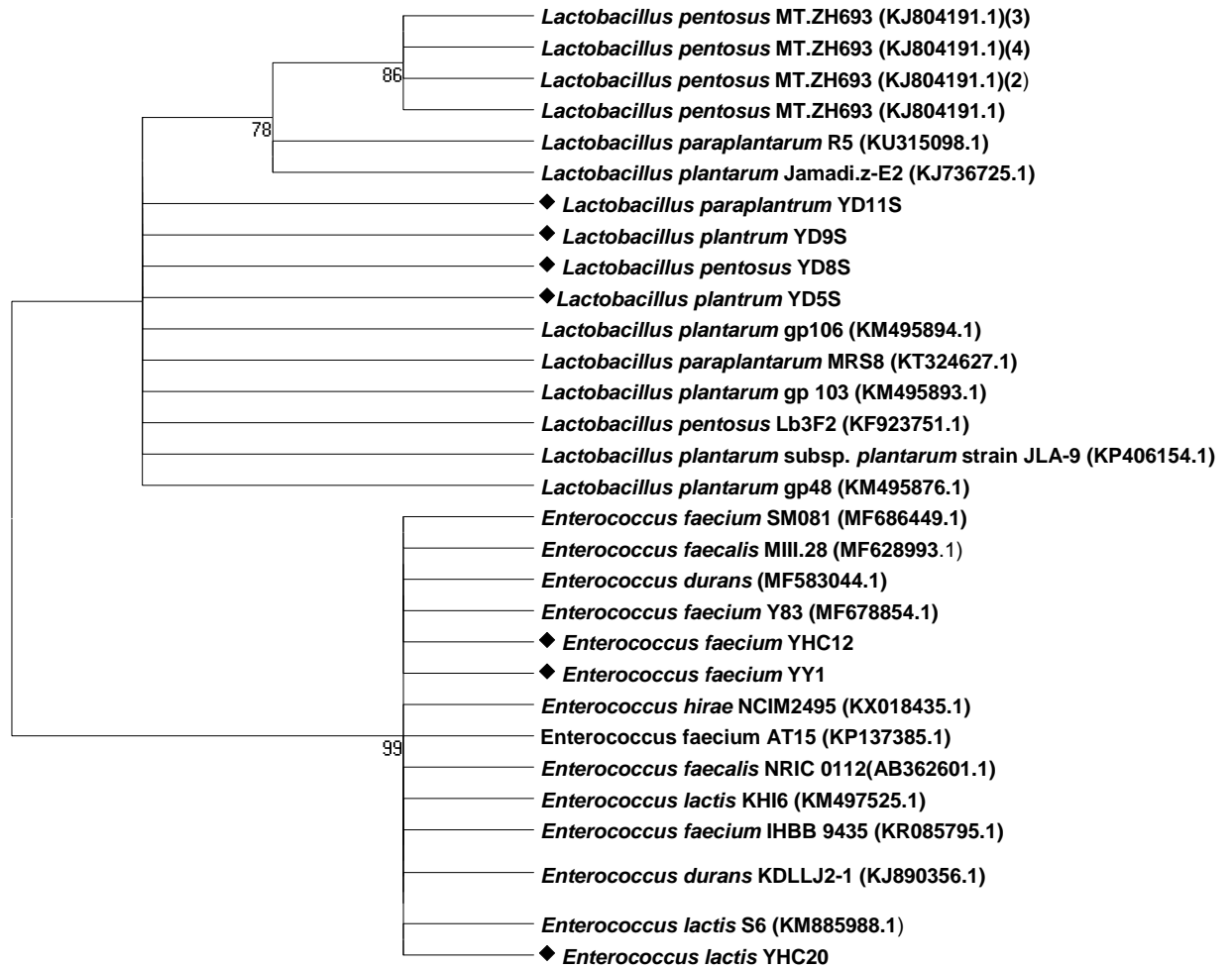
**Figure 24:** Evolutionary relationships of taxa of LAB strains fermented yak milk product. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.09376396 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.
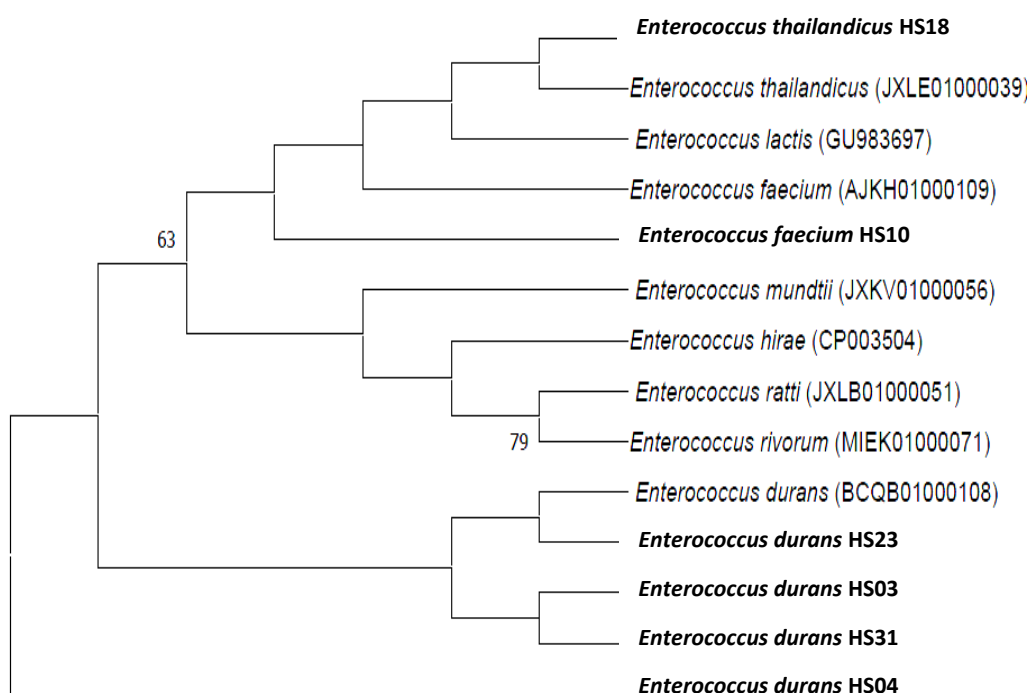
**Figure 25:** Evolutionary relationships of taxa of LAB strains from human fecal sample

The sequences belong to two clusters of Lactobacillaceae family and another to Enterobacteriaceae family. Similarly the phylogenetic tree for human faecal strains was constructed where the bootstrap values were evaluated on the basis of 1000 replicates shown in Figure 25. There was a single cluster belonging to the Enterobacteriaceae family. The sequence for *Enterococcus faecium* HS44 was not used in constructing the tree as it was only 485 bp.

### 4.11. Antibiotic succeptibility of *Enterococcus* strains

For susceptibility testing, 7 antibiotics were tested namely; erythromycin 15mcg, streptomycin 10 µg, gentamycin 120 µg; penicillin 10 µg, tetracyclin 30 µg,

chloramphenicol 30 µg and kanamycin 5 µg. The antibiotic susceptibility of *Enterococcus* strains against seven antibiotics have been presented in Table 30. The *E coli* MTCC 1098 and *Staphylococcus aureus* MTCC 7443 were used as control strains for antibiotic susceptibility. The zone of inhibition obtained was matched with Clinical and Laboratory Standards Institute, CLSI (2014) for reference strain. The *E coli* ATCC 235922 and *Staphylococcus aureus* ATCC 235923. The zone of diameter range for susceptibility was 21-31mm, 16-20mm for intermediate and 0-15mm for resistance as described by Volkova et al. (2008) and Chartiers et al. (1998). The largest zone of inhibition was observed as 28mm for erythromycin shown by *Enterococcus faecium* HS04. All the isolates were sensitive to erythromycin with the zone of inhibition ranging from 24mm-28mm. Isolates were resistant to streptomycin, except *Enterococcus faecium* HS04 and *Enterococcus faecium* HS31 showing 16mm zone with intermediate susceptibility. For gentamycin *Enterococcus faecium* HS18, *Enterococcus faecium* YHC20 and *Enterococcus faecium* HS44 gave intermediate susceptibility while remaining showed sensitivity.

All isolates showed resistance to kanamycin and penicillin, sensitivity towards tetracycline and intermediate and sensitive patterns towards chloramphenicol. Thus strains were 100% susceptible to erythromycin and tetracyclin and 100% resistance to kanamycin and penicillin.

In E-test the minimum inhibitory concentration (MIC) for six antibiotics was done using paper strips that had antibiotic concentration gradient from 0.016mcg/ml to 256mcg/ml on the *Enterococcus* isolates. The results of MIC are presented in Table 31.

**Table 30:** Antibiotic sensitivity pattern of *Enterococcus* sp.

| Source | Strain | MIC µg/ml | | | | |
|---|---|---|---|---|---|---|
| | | **E** | **S** | **GEN** | **TET** | **C** |
| Fermented yak milk | *Enterococcus lactis* YY1 | 24-S | 13-R | 14-R | 23-S | 20-S |
| | *Enterococcus faecium* YHC20 | 25-S | 10-R | 16-I | 24-S | 21-S |
| | *Enterococcus faecium* YHC12 | 26-S | 13-R | 10-R | 25-S | 25-S |
| Human fecal | *Enterococcus durans* HS03 | 24-S | 11-R | 15-R | 26-S | 24-S |
| | *Enterococcus durans* HS04 | 28-S | 16-I | 12-R | 23-S | 18-I |
| | *Enterococcus faecium* HS10 | 24-S | 13-R | 10-R | 26-S | 23-S |
| | *Enterococcus* sp HS18 | 26-S | 11-R | 16-I | 23-S | 25-S |
| | *Enterococcus durans* HS23 | 25-S | 13-R | 14-R | 26-S | 20-S |
| | *Enterococcus durans* HS31 | 26-S | 16-I | 10-R | 26-S | 26-S |
| | *Enterococcus faecium* HS44 | 24-S | 10-R | 16-I | 25-S | 25-S |

Note: E, Results were expressed as sensitive, S (≥21mm), intermediate, I (16-20mm) and Resistant, R (≤15mm) respectively according to that described by Volkova et al. (2008) and Chartiers et al. (1998). The isolates were resistant to Penicillin and Kanamycin.

**Table 31:** Determination of Minimum inhibitory concentration (MIC) of *Enterococcus* strains

| Source | Strain | Antibiotics | | | |
|---|---|---|---|---|---|
| | | **E** | **GEN** | **TET** | **C** |
| Fermented yak milk | *Enterococcus lactis* YY1 | 0.5 | 8 | 6 | 2 |
| | *Enterococcus faecium* YHC20 | 0.5 | 8 | 6 | 2 |
| | *Enterococcus faecium* YHC12 | 0.5 | 12 | 0.38 | 1. |
| Human fecal | *Enterococcus durans* HS03 | 1 | 8 | 0.38 | 2 |
| | *Enterococcus durans* HS04 | 0.5 | 16 | 0.38 | 2 |
| | *Enterococcus faecium* HS10 | 4 | 24 | 0.19 | 1.5 |
| | *Enterococcu thailandicus* HS18 | 0.75 | 8 | 6 | 2 |
| | *Enterococcus durans* HS23 | 1 | 17 | 6 | 4 |
| | *Enterococcus durans* HS31 | 0.5 | 32 | 6 | 8 |
| | *Enterococcus faecium* HS44 | 0.5 | 12 | 0.19 | 2 |

Since the isolates were resistant to Penicillin and Kanamycin, the MIC was not determined for these antibiotics.

# Discussion

## 5. Discussion

CVD is a major cause of premature death in the world. In 2015 CVD accounted for about one-third of all deaths alone, and there was an estimated rate of 422 million prevalent cases (Roth et al., 2017). There are about 300 risks associated with CVD; one of the most significant is increase in abnormal blood lipids including LDL-cholesterol, triglycerids, high total cholesterol and low levels of high density lipoprotein (HDL) cholesterol. With increasing prevalence of CVD and the absence of a safe therapy, a novel therapeutic representative has become important.

The present study aimed to screen LAB for its cholesterol lowering and probiotic property. A total of 160 isolates from fermented yak milk products and 60 healthy human fecal isolates were isolated, preliminary characterized and screened for cholesterol lowering property, additional probiotic characteristics were studied, the mechanisms of cholesterol lowering was determined and the antibiotic susceptibility was tested then the selected isolates were then identified by sugar fermentation and 16S rRNA gene sequencing.

### 5.1. Isolation and preliminary characterization of lactic acid bacteria (LAB)

The novel strains with cholesterol lowering property had been obtained from two different sample sources; fermented yak milk products like *Chhurpi*, *Shyow*, *Thara* or *Khachu* and the healthy human feces. Fermented yak milk has been found to be potential sources of probiotics recently (Ding et al., 2017; Kaur et al., 2017). 160 fermented yak milk isolates and 60 human fecal isolates were preliminary tested for presence of catalase, Gram staining nature, KOH test, and cell morphology and grouped into rods and coccus. Similarly in a study; based on the positive Gram reactions, absence of catalase, lack of motility, cell morphology and the presumptive

LAB isolates were grouped as cocci (61% in total) and rods (39% in total) reported to be obtained from *kurut*, *qula* cheese, raw milk, whey, and butter made from yak milk in China (Bao et al., 2012).

## 5.2. Screening of the isolates for cholesterol lowering

A probiotic strain should also have at least one health benefit. All the isolates were studied for cholesterol lowering which was determined spectrophotometerically similar to the method used by Pereira & Gibson (2002). The cholesterol lowering or reduction percentage ranged from 15 to 90% shown by our isolates in MRS broth containing water soluble cholesterol. >50% cholesterol reduction was shown by 23% of fermented yak milk product isolates and by 57% human fecal isolates. The number of cholesterol reducing isolates is more from fecal samples than from the yak milk product. This could be due to differences in the type of isolates and ecological niche where they are found. The environments where the gut bacteria live are frequently exposed to dietary cholesterol as well as bile salts and hence most of the fecal isolate are able to reduce cholesterol. Nevertheless some isolates from yak milk products are also efficient in reducing cholesterol. In fact some yak milk LAB isolate can reduce cholesterol more than fecal isolates. A recent study reported cholesterol reduction in MRS medium upto 45.84% by a *Lactobacillus* sp. strain HLX37 (Guan et al., 2017). 0.4 to 47% of cholesterol lowering in the media was earlier reported by Pereira & Gibson (2002). A previous study showed that some *Lactobacillus reuteri* strains reduced cholesterol from 20.18 to 59.94%, and some *Lactobacillus acidophilus* strains reduced the level from 49.57 to 55.43% within 24 h (Al-Saleh et al., 2006; Tomaro-Duchesneau et al., 2014). In another study, *Lactobacillus casei* and *Lactobacillus sakei* reduced the level of cholesterol by about 30% and *Pediococcus acidilactici* by 20% within 24 h (Tsai et al., 2014; Song et al., 2015). *Enterococcus*

*durans* KLDS 6.0930, *Enterococcus durans* KLDS 6.0933, *Enterococcus faecalis* KLDS 6.0934 and *Enterococcus faecalis* KLDS 6.0935 isolated from a traditional naturally fermented cream in China were reported to reduce cholesterol in medium by 56.61%, 46.99%, 41.29% and 52.01% respectively (Guo et al., 2016).

Those isolates giving $\geq$ 50 % cholesterol lowering was studied for probiotic characteristics like tolerance to acid, bile salt and cell surface hydrophobicity to check their viability and tolerance in the human GI tract.

## 5.3. Additional probiotic attributes

The most important selection criteria for potential probiotic microorganisms are acid or low pH tolerance, bile salt tolerance and cell surface hydrophobicity. Therefore, it was essential to check if the cholesterol reducing isolates could overcome stress from acidic conditions prevalent in the stomach and bile salt in the intestine. The LAB entering the body through oral administration must pass through the highly acidic (approximately pH 3) gastric fluid in the stomach and the weakly basic (pH of 7.8–8.4) intestinal juice that contains 0.3–2% (w/v) bile-salts in the upper portion of the intestinal tract where they can stay for 1–2 h (Navarre & Schneewind, 1999; Chen et al., 2010). It is estimated that the time from the consumption of food to its release in the stomach is 90 min (Berada, 1991). The pH of gastric juice secreted in the stomach is very low and many microorganisms are destroyed at this lower pH. The prevalence and viability of a probiotic microorganism in the gastrointestinal tract is one of the important features as to exert beneficial effects. Acid tolerance enables strains to survive for longer period of time in high acid carrier food (Conway et al., 1987; Prasad et al., 1998), thus making it a very important criterion (Huang & Adams, 2004) for selection of probiotic bacteria. For the selection of acid tolerance pH 2.0 is thought

as a strong differential pH (Turchi et al., 2013). All the isolates remained viable when exposed to pH 2.5 for 1 h though their viability decreased to various levels. Isolates from fermented yak milk products and human feces also remained viable up to 2 h at pH 2.5. Few of our isolates were also able to tolerate pH 2 for 2 h. The results were in agreement to the results reported by Liong & Shah (2005) where *L. acidophilus* ATCC 4962, *Lactobacillus acidophilus* ATCC 4357, *L casei* ASCC 290 and *L casei* ASCC 290 survived best at pH 2. The decline of cell viability of LAB at such a low pH was also reported by Raghavendra et al. (2010). All the isolates showed different tolerance at varying pH. In a study *Lactobacillus* sp strain HLX37 was acid tolerant, at simulated gastric juice (pH 2.5) for 2 h. Specifically, in simulated gastric juice (pH 3) or in acid (pH3.0) for 2 h, the survival rates were 33.41% or 85.45%, respectively, while human gastric juice the rates were 15 and 45% (Guan et al., 2017). Similar results were obtained at pH 2.5 where some isolates showed reduced viability that were similar to the results obtained in *L. casei and Enterococcus* sp. (Nagata et al., 2009; Lidong et al., 2016). Acid resistance differ among different species of lactic acid bacteria and is believed to be related to proton pump mechanism, its reaction mechanism, macromolecular protection and repair, alkali generation, regulators, cell density and biofilm formation may also affect the acid resistance of lactic acid bacteria (Chen et al., 2008).

Another hurdle for survivability of a potential probiotic LAB is tolerance to bile salts in the small intestine. Bile salts are thus toxic to living cells and are regarded as one of the major constituents of bile capable of disrupting the structure of cell membranes (Begley et al., 2006). Tolerance to bile helps LAB to reach the small intestine and finally to colon which give an added advantage in balancing the intestinal microflora (Tambekar & Bhutada 2010). Liong & Shah (2005) had used 0.3% of three different

bile salts sources, oxgall (mixed bile) taurocholic acid (conjugated bile) and cholic acid (deconjugated bile). However, in this study two different concentrations of bile salts (oxgall, taurocholic acid and cholic acid) 0.5% and 1 % for 0 h, 4 h and 8 h were considered since it has been reported by Dawson (1998) that the bile salt concentration in the small intestine ranged from approximately 0.2 to 2% (wt/vol). The bile salt concentration is known to depend on the person, type and amount of food ingested (Dawson, 1998).  There are other reports which mention human bile as 0.5% (Mathara et al., 2008), 0.1 to 0.3% (Dunne et al., 2001) and the prevailing time is suggested to be 4 h (Mishra & Prasad, 2005). At the first hour of digestion bile salt is detectable at the level 2% and it gradually decreases to 0.3% during time of prolonged digestion (Noriega et al., 2006). The capability to tolerate bile salts in MRS broths with 1.0, 0.5 and 0.3 % in forty-two *Lactobacillus plantarum* strains isolated from different Italian food sources (milk, cheese, fermented meat products) was reported by Turchi et al. (2013). In a study conducted by Mathara et al. (2008), *L. plantarum* strains isolated from fermented milk products could tolerate 0.1–0.5% bile salts that were similar to our results at 0.5% bile concentration. Our LAB strains grew in MRS broth supplemented with taurocholic acid may be due to the presence of bile salt hydrolase enzymes that are able deconjugate the conjugated bile (Moser & Savage, 2001; Taranto et al., 2006). The BSH activity of the isolates revealed the ability to hydrolyze bile salt causing cholesterol precipitation, thus enhancing the viability of bacteria even under toxic bile salt condition (Pisano et al., 2008). The resistance towards conjugated bile in some bacteria may be because of detergent activity (Liong & Shah, 2005), greater solubility and presence of efflux pump and BSH activity (Bustos et al., 2011).

The three *Lactobacillus* strains isolated from infant feces and pickled vegetables showed greater reduction in viability at 1% bile salt when compared with 0.3% bile salt Wang et al. (2016). It is believed that the increase in bile concentration decreases the survival rate due to change in membrane permeability and membrane protein dissociation that eventually lead to intracellular material flow causing some cell death (Begley et al., 2005). However the present study suggests that LAB isolates from fermented yak milk product and human feces can tolerate low pH as well as bile salt at a concentration in the human GI tract.

The bacterial affinity towards the hydrocarbons proves the ability of the LAB to anchor to surface of host cells determining its colonization ability, which is a very important step in the establishment of probiotics in the gut (Orlowaski et al., 2006). Boris et al. (1998) considered isolates having hydrophobic index >40% as hydrophobic while Nostro et al. (2004) recommended that hydrobhobicity index >70% to be considered as hydrophobic for adherence to the cell surfaces. Our isolates have shown hydrophobocity percentage in the range of 20.50% to 94.80 by fermented milk isolates and 6.67% to 96.61% by human isolates, out of which 16 isolates have shown above 40%. The higher the values of cell surface hydrophobicity the greater will be the ability of a bacterium to attach or stick on the cells of the epithelial layer of the intestine (Rosenberg et al., 1984). As reported by Tamang et al. (2009) the LAB strains *Lb. brevis* strains MeN7 (BFE942), MeR6 (BFE938), SL: B7 (BFE2889), KG: B2 (BFE952), MeTR (BFE941) and *Lb. plantarum* strains MeL2 (BFE934) and MeL3 (BFE944) showed more than 70% hydrophobicity. Similarly the results for hydrophobocity in n–hexadecane ranged from 37.80% - 85.67%, 21.06 % - 88.00% and 76.33 %, for *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Lactobacillus brevis* respectively (Jamaly et al., 2011). In another study the hydrophobicity values

for probiotic strains, ranged from 38.1 to 67.8% by *L. acidophilus* (Vindderola, 2003). 50 strains of *Lactococcus lactis* that were studied for hydrobhobicity revealed very high values ranging reaching 48–88% (Giaouris et al., 2009). Different strains of species may vary with respect to the affinity towards hydrocarbons which may be due to the cell surface proteins (Ramiah et al., 2007). However, the adherence property varies at strain level as reported in different *in vivo* experiments.

## 5.4. Mechanism of cholesterol lowering

In the present study the some of the LAB isolates from fermented milk product  and human feces sample such as  *Pediococcus* YHC6    (90%),    *Lactobacillus*    YD5S (85%), *Lactobacillus* YW45 and *Pediococcus* YW35 (80%), *Lactobacillus* YHC12 (80%), *Enterococcus* YD12S (80%), *Lactobacillus* YD15S (75%), *Lactobacillus* YD9S (70%), *Lactobacillus* YD7S (70%)   and human fecal isolates; *Enterococcus* HS03 (70%), *Enterococcus* HS06 (70%), *Enterococcus* HS08 (70 %), *Pediococcus* HS22 (70%), *Enterococcus* HS23 (70%), *Enterococcus* HS29 (70%), *Enterococcus* HS40 (70%), *Enterococcus* HS43 (70%), and *Enterococcus* HS44 (70%) showed maximum cholesterol lowering activity upto 90% in 24 h which may be the important finding.

It has been reported that the ability of  a microorganism to reduce the cholesterol level was due to cholesterol assimilation (Rasic et al., 1992; Piston & Gilliland, 1994; Pereira & Gibson, 2002), cholesterol co-precipitation with deconjugated bile,  binding of cholesterol to the bacterial cell wall (Hosono & Tono-oka 1995; Kimoto et al., 2002), and enzymatic deconjugation of bile acids due to the presence of BSH enzyme (Sugano, 1986; De Smet et al., 1995; Salminen et al., 2002; Liong et al., 2005; Lye et al., 2010). The isolates showing greater cholesterol lowering activity with additional

property of tolerance to acid, tolerance to bile and hydrophobic nature were further investigated to find out the possible mechanisms of cholesterol lowering activity.

## 5.4.1. BSH activity

An opaque white precipitation was observed on streaking of the LAB isolates in MRS agar plates supplemented with taurodeoxycholic that were indicative of positive result, and depending on the extent of precipitation the isolates were categorised into high, intermediate and low. BSH enzymes are intracellular enzymes that cause hydrolysis of the amide bond between a steroid moiety and amino acid side chain of the bile acids (Lebeer et al., 2008). Substrate specificity, optimal temperature and pH for enzymatic activity of BSH enzyme differ from various sources. Begley et al. (2006) also reported that BSH activity is not detected in bacteria isolated from environments from which bile salts are absent. In 2015 there was the first report on BSH activity over primary bile salts by *L. casei* isolated from pulque, a fermented beverage produced by the fermentation of agave sap (González-Vázquez et al., 2015). It was also noted that glycine conjugates had a stronger effect over survival of *Lactobacillus casei* strain. However, Archer& Halami (2015) reported isolates from both human feces and dairy sources were found to show precipitation and growth in TDCA agar plates showing their ability to produce bile salt hydrolase enzyme which were in accordance to our result. Out of 69 LAB isolates screened for the production bile salt hydrolase (BSH), 21 isolates from fermented yak milk sample and 31 from human feces sample gave positive BSH activity, out of which those showing heavy precipitation were studied further.

The bile salt hydrolase enzyme is active on both glycine and taurine conjugated bile salts. On determining the BSH activity quantitatively our isolates

showed a varying degree of substrate preference towards sodium glycocholate and sodium taurocholate. In humans where glycine: taurine ratio in humans is 3:1 (Vlahcevic 1990), the conjugation of taurine is higher depending on diet rich in animal protein while there is conjugation of glycine in individuals with vegetarian diets (Sjövall, 1959; Hardison, 1978).

In some experiments performed by Corzo & Gilliland (1999) where glycocholate to taurocholate ratio was 2:3 at pH 6.5, *L. acidophilus* efficiently deconjugated glycine conjugated bile salt than taurine conjugated bile salt. At a pH of 5.50-6.50 resembling the pH of upper intestinal tract around half of free bile salts and a small amount of glycine-conjugated bile salts were found to be protonated or nonionized, while no protonation occurred in bile salt that was taurine-conjugated (Carey & Cahalane, 1988); this is because the pKa values of taurine and glycine conjugated bile salts, and of deconjugated bile salts are 1.9, 3.9 and 5.0 respectively. Therefore, at acidic pH, deconjugated bile salts are nonionized and gets precipitated, taurine-conjugated bile salts remain ionized in solution while glycine-conjugated bile salts are moderately precipitated without hydrolysis (Dashkevics & Feighner, 1989).

The highest total activity with glycine conjugated bile was observed in *Enterococcus* YY1 (1.30 U/ml), and with taurine conjugated bile by *Enterococcus* YHC8 and *Lactobacillus* YW45 (1.11 U/ml). Most of our isolates showed preference towards SG, that were similar to the earlier reports of identified BSH enzymes having a narrow substrate spectrum and a much higher activity in hydrolyzing glycine conjugated bile salts than taurine-conjugated bile salts (De Smet et al., 1995; Coleman & Hudson, 1995; Tanaka et al., 2000; Kim et al., 2004; Liong & Shah, 2005; Pavlović et al., 2012). However some of our isolates, *Lactobacillus* YW45 (1.11 U/ml), *Enterococcus* YHC8 (1.11 U/ml) and *Lactobacillus* YD9S (1.06 U/ml) showed more

preference to taurine conjugated bile and there has been as in earlier reports of BSH enzymes of *Lactobacillus* showing more affinity towards taurine-conjugated bile salts (Chae et al., 2013). In glycine conjugated bile salt, glycine is assimilated to ammonia and carbon dioxide by some bacteria and taurine is assimilated to ammonia and carbon dioxide along with the release of sulfite. However, it has also been observed that BSH positive *Clostridium* sp. was able to utilize taurine resulting in improved growth by Stickland fermentation (Huijghebaert & Eyssen, 1982). *Bacteroides* metabolized taurine from TCA by stimulating to cholic acid 7α-dehydroxylation through the release of sulfite indicating the end-product metabolism would further enhance degradation of bile salt (Van Eldere et al., 1996). In a study involving *B. longum* BBMN68 it was reported that this strain expressed a hemolysin-like protein that provided a greater tolerance to taurine-conjugated bile acids but not glycine-conjugated bile acids similar to our strains showing affinity to taurine conjugated bile (Liu et al., 2014).

Some studies also pointed out the survival of LAB in bile and BSH activity cannot be correlated (Zago et al., 2011; Solieri et al., 2014) while other studies has reported that the BSH activity increases tolerance to bile salt (Noriega et al., 2006; Burns et al., 2010). The ability to bring about bile salt hydrolysis may contribute in the survival and progression of bacterial strains in the intestinal tract, as earlier shown for *Listeria monocytogenes* (Dussurget et al., 2002). In a study involving *L. plantarum* WCFS1 strain, it was capable of persisting in gastrointestinal tract of mouse for 10 days (Pavan et al., 2003) and it has also displayed considerably high tolerance and activity during passage of the human gastrointestinal tract (Vesa et al., 2000).

## 5.4.2. Bile acid deconjugation and co- precipitation of cholesterol

Further the isolates were tested for their deconjugation ability on taurine and glycine conjugated bile types. The bile acid deconjugation was recorded on the discharge of cholic acid from the hydrolysis of conjugated bile. The free bile acids formed by the deconjugation of conjugated bile salts are less soluble and are have low chance of likely being reabsorbed by the intestinal cells compared to their conjugated portion, that are lost from the body through feces (Center, 1993). This leads to a higher demand of bile acid synthesis utilizing the cholesterol from the serum and consequently, the reduction of serum cholesterol (Reynier et al., 1981). In our results glycine conjugated bile salt was more actively deconjugated than taurine-conjugated bile salt which also supports our data that BSH activity was more towards SG than towards ST which is similar to the results of Liong & Shah (2005) as substrate specificity was more towards glycine-conjugated bile. *Lactobacillus* YD8S (3.30 mM), *Lactobacillus* YD5S (2.47 mM), *Lactobacillus* YD9S (1.97 mM) were the highest cholic acid released through deconjugation of glycine conjugated bile which were in similar to previous experiments of Corzo & Gilliland (1999), where glycine conjugated bile salt was more efficiently deconjugated than taurine conjugated bile salt by strains of *L. acidophilus* from both human and porcine origins at pH of 6.5.

It was hypothesized that when deconjugated bile salts and pH of the media dropped as a result of acid production, the cholesterol micelles get destabilized and cholesterol get co-precipitated (Klaver & Van der Meer 1993). Co precipitation was calculated by measuring the difference between final cholesterol present in the MRS broth inoculated with culture and the uninoculated MRS broth with cholesterol that served as the control. Due to deconjugation of bile salt by the BSH enzyme and increase in pH of the broth, the bile salts are precipitated while taurine conjugated bile salt

remain ionized in solution, and glycine conjugated bile salt are partially precipitated without hydrolysis (Dashkevicz & Feighner 1989). As a result of bile salt deconjugation, the amino acids liberated could possibly utilized as carbon, nitrogen, and energy sources, as glycine may be metabolized to ammonia and carbon dioxide, and taurine may be metabolized to ammonia, carbon dioxide, and sulphate. Thus deconjugation of bile salt give an added advantage on nutrition to those strains showing hydrolysis (Begley et al., 2006). This was supported by the experiment on *Clostridium* sp. that used taurine obtained from deconjugation as a terminal electron acceptor and the growth rates were reported to improve in presence of taurine and taurine conjugated bile salt (Van Eldere et al., 1996).

### 5.4.3. Incorporation of cholesterol into the cellular membrane

Some of our LAB isolates revealed cholesterol lowering but were not BSH active. Therefore, the presence of BSH enzyme leading to deconjugation or coprecipitation of bile salt may not be fully responsible for the cholesterol-reducing activity. There were earlier reports from studies by Hosono (1999) that out of 28 *L. gasseri* 27 were able to remove cholesterol from the medium without BSH activity. Thus there might be some other mechanism involved in cholesterol removal from the media. Some workers suggested that *in vitro* removal of cholesterol was due to assimilation of cholesterol (Gilliland et al., 1985) or cholesterol incorporation into the cellular membrane (Noh et al., 1997; Brashears et al., 1998; Liong & Shah, 2005). Our non BSH active LAB isolates were studied for *in vitro* cholesterol incorporation into the cellular membrane. The results of cellular cholesterol ranged from 10-72%, the highest shown by *Enterococcus* YHC12 and the lowest by *Lactobacillus* YD6S. The degree of cholesterol incorporation was isolate dependent. In a study reported by Noh et al. (1997), *Lactobacillus acidophilus* ATCC 43121 showed assimilation, and cholesterol

could be obtained from their membranes even after sonication causing a possible alteration of the cell wall or membrane.

### 5.4.4. Cholesterol assimilation

In a study conducted by Dambekodi and Gilliland (1998), no link was observed between the amount of cholesterol removed and deconjugation of bile salt, this lead to the hypothesis of cholesterol assimilation. No cholesterol assimilation was observed in *L. acidophilus* strains ATCC 43121 and NCFM-L strains under frozen storage condition when tested for their abilities to tolerate bile and cholesterol assimilation (Piston & Gilliland 1994). It was concluded that the decrease in activity during refrigerated storage condition caused a decrease in activity at *in-vivo* conditions and declared that cholesterol assimilation activity is growth dependent. Our results of cholesterol assimilation were in the range of 21% to 93% in MRS broth supplemented with cholesterol and 17% to 90% in the same broth with ox bile. Cholesterol assimilation as high as $59.94 \pm 7.49$ $\mu$g/ml by *L. reuteri* NCIMB 702656 was reported earlier (Tomaro-Duchesneau et al., 2014). Cholesterol assimilation percentage decreased in MRS containing 0.3% bile and cholesterol in this study , however earlier studies have shown that, in order to assimilate cholesterol, the organisms must be able to grow in the presence of bile salts (Gilliland et al., 1985; Tahri et al., 1995; Tahri et al., 1996; Walker & Gilliland, 1993; Dambekodi & Gilliland, 1998). There were some exceptions in case of *L. crispatus* ATCC 33820, where other cultures exhibited some degree of bile tolerance (Usman & Hosono, 1999). However it was found that the strains with high bile tolerance did not essentially assimilate more cholesterol than those with a lower tolerance.

## 5.5. Bacterial identification by Sugar fermentation and 16S rRNA sequencing

The cholesterol lowering LAB isolates were identified according to Axelsson (2004) upto genera level. The isolates were then tested for its probiotic characteristics and possible mechanisms. Then sugar fermentation tests were conducted to determine the identity of these probiotic isolates till species level. Sugar fermentation profile indicated that these LAB isolates from fermented yak milk and human feces belonged to *Lactobacillus* sp. (YD8S, YD5S, YD9S, YD11S) as *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, and *Enterococcus* sp. (YY1, HS03, HS18, YHC20, HS23, HS04, YHC12, HS10, HS31, HS44) as *Enterococcus lactis*, *Enterococcus faecium* and *Enterococcus durans* etc. Final identity was confirmed using 16S rRNA gene sequencing. Till date many workers have been using 16S rRNA gene sequencing for identification of LAB. For bacterial identification molecular methods are important (Sghir et al., 2000; Greetham et al., 2002; Heilig et al., 2002; Pavlova et al., 2002; Tamang et al., 2008; Liu et al., 2012; Archer & Halami, 2015; Wang et al., 2016) and probably more accurate for LAB than the conventional phenotypic methods. However, in 1987 Woese first proposed the identification of microorganisms by their 16S rDNA region. The 16S rRNA gene or region remains highly conserved and this region, 16S or 23S rRNA can be targeted by oligonucleotide primers making this identification system as one of the best and most accurate approaches to bacterial identification to draw phylogenetic relationships (Charteirs et al., 1997; Rolfe, 2000).

LAB from cow and yak milk products of Sikkim on the basis of phenotypic characters were earlier reported as *Lactobacillus alimentarius*, *Lb. farciniinis*, *Lb. salivarius, Lb. bifernientans*. *Lb. brevis* and *Lactococcus lactis* sub sp. *cremoris* by Dewan & Tamang (2007). Similarly, In 2009 LAB from Tibetan yak milk products

were identified phenotypically using assimilation and fermentation kits API 50 CH as *Lactobacillus fermentum*, *Lactobacillus helveticus* and *Lactobacillus curvatus* (Wu et al., 2009) which were also similar to the results of Miyamoto et al., (1989). The predominant populations in the yak milk products were *Lactobacillus helveticus* and *Lactobacillus casei* in China (Bao et al., 2012). *Enterococcus durans* (45.3%), *Lactobacillus fermentum* (22.6%), and *Lactobacillus paracasei* (17.0%) were the dominant LAB strains reported in *Xueo*, the fermented yak milk products in the western Sichuan Plateau of China identified by 16S rRNA gene sequencing (Ao et al., 2012). Yak milk products namely (*kurut*, *qula* cheese, raw milk, whey, and butter of Sichuan province of China were studied for the diversity of LAB by phenotypic characterization, 16S rRNA gene sequence analysis, species-specific PCR, and PCR-RFLP (Bao et al., 2012). The results revealed the presence of *Leuconostoc* (40.8%), *Lactobacillus* (39.0%), *Streptococcus* (13.2%), *Lactococcus* (5.6%), *Enterococcus* (0.94%), and *Weissella* (0.46%), which were similar to our results that revealed the presence of *Lactobacillus* sp., *Lactococcus* sp. and *Enterococcus* sp. In a previous study using high-throughput sequencing; *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Luteococcus*, and *Streptococcus* were detected in the Tibetan traditional fermented yak milk by using a culture-independent method (Liu et al., 2015). Since in this study only cholesterol reducing bacteria, both BSH active and non active and other probiotic characteristics were considered, 14 identified strains were identified by 16S rRNA gene sequence analysis as *Lactobacillus pentosus* YD8S, *Lactobacillus plantrum* YD5S, *Lactobacillus paraplantrum* YD11S, *Lactobacillus plantrum* YD9S, *Enterococcus faecium* YY1, *Enterococcus lactis* YHC20, *Enterococcus thailandicus* HS18, *Enterococcus faecium* HS10, *Enterococcus durans*

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HS23, *Enterococcus durans* HS31, *Enterococcus durans* HS04, *Enterococcus faecium* YHC12, *Enterococcus durans* HS03 and *Enterococcus faecium* HS44.

There were many studies where Yak milk products like yogurt were reported to contain a higher amount of LAB when compared to other cattle milk (Guo et al., 2014). Yak milk has high nutritional value including rich fats like polyunsaturated fatty acids (PFA) like conjugated linoleic acid and omega three fatty acids along with proteins and the essential minerals content (Luming et al., 2008; Liu et al., 2011). In a study, antioxidant activity with tolerance to acid and bile was studied in LAB isolated from fermented yak milk collected on the Tibetan Plateau (Ding et al., 2017). A recent study evaluated to screen cholesterol-lowering lactic acid bacteria from traditional fermented Tibetan yak milk, for its use as probiotics in rats (Ding et al. 2017).

In a recent study, LAB was isolated from the natural fermented yak yogurt from Yushu area of Gansu province, China using the traditional combination of physiological and biochemical experiments and 16S rDNA gene sequence analysis the strain was identified as *Lactobacillus plantarum* YS2, similar to our result. This strain showed high tolerance to acid and bile along with high hydrobhobicity percentage (Qian et al., 2018).

*Lactobacillus* strains namely *L. rhamnosus*, 4B15 and *L. gasseri* 4M13, isolated from infant feces were reported to show cholesterol lowering and possess probiotic property (Oh et al., 2018). In another study isolation and evaluation of some probiotic lactic acid bacteria from infants stool was evaluated with an aim to degrade and reduce cholesterol level in fermentation medium. One strain that gave 71.6% as the highest cholesterol reduction percentage was identified by 16S rRNA gene

sequencing as *Enterococcus faecium* which was in accordance to our results of cholesterol lowering fecal strain identified as *Enterococcus faecium* HS44 and *Enterococcus faecium* HS10.

## 5.6. Antibiotic resistance of *Enterococcus* sp.

For considering the genera *Enterococcus* sp. as a probiotic, a number of criterias are contemplated, especially those belonging to the host microbiota which is being considered for oral administration, it has to be non pathogenicity and there should be absence of antibiotic resistance genes (Saarela et al., 2000). Many studies have been based on the use of *Enterococcus* sp. as probiotic. A recent study investigated *Enterococcus faecium* CCDM 922 from the Culture Collection of Dairy Microorganisms Laktoflora® for technological properties including antibiotic resistance and cholesterol lowering activity *in vivo* (Hyrslova et al., 2016). *Enterococcus* sp. is commensal, capable of causing the disease especially in immune compromised patients but they do not secrete any potentially virulent toxin. They may cause infection from the intestine of an individual and may also be able to transfer infection to another healthy individual through contaminated food or water (Brilliantova et al., 2010).

Enteroccci are able to transfer the antibiotic resistance genes to produce β-hemolysin (Franz et al. 2001), gelatinase (Huycke et al., 1991) and clumping substance (Sartingen et al., 2000) that are unwanted characters in probiotic strain. They are even capable of acquiring resistance genes (on plasmids or transposons) from other microorganisms, with higher probability of acquiring the resistant pathogenic markers than other bacteria in the same habitat (Chopra & Roberts, 2001). The probiotic strains are hence needed to show susceptibility to antibiotics because

antibiotic resistance determinants can transfer horizontally to pathogens in the gut (Gueimonde et al., 2013).

In our study the identified *Enterococcus* sps were further studied for antibiotic sensitivity. It was found that *Enterococcus durans, Enterococcus faecalis* and *Enterococcus faecium* were predominantly found in healthy human feces (Barreto et al., 2009). More than one-fourth (28.7%) of the isolates were resistant to tetracycline; 21.8% were resistant to erythromycin and 8.9% were resistant to kanamycin, which revealed that enterococci of healthy growing children's could be a reservoir of antimicrobial resistance genes (Barreto et al., 2009). Similarly in another study where antibiotic susceptibility was tested in 73 *Enterococcus faecalis*, 45 *E. faecium* and 22 of other species) from fecal samples of wild animals in Portugal revealed 44 isolates (31.4%) showing susceptibility to all the antibiotics tested which was in accordance to our isolates showing susceptiblity to erythromycin, tetracyclin and chloramphenicol. Our isolates showed resistance to kanamycin and penicillin unlike the results where isolates showed ampicillin and vancomycin resistance but tetracycline and erythromycin resistances were shown in 28.6% and 20.1% of the isolates, respectively (Poeta et al., 2005). The resistance to kanamycin and penicillin may be plasmid borne or associated with the chromosomal DNA. However, our probiotic *Enterococcus* sp. have to be checked for *in vitro* and *in vivo* conjugation (Lund & Edlund, 2001) and should be considered for evaluations for the safety of enterococcal probiotics.

# Summary

**Summary**

The purpose of the study was to isolate lactic acid bacteria (LAB), screen for cholesterol lowering, and characterize them based on probiotic nature and study the mechanisms of cholesterol lowering. Samples of *Thara* (Mohi in Nepali), *Shyow* (Dahi in Nepali)*, Hard and soft type *Chhurpi*, and *Marr* (Ghew in Nepali), the fermented yak milk products were collected from 9th mile and Tsongmo (East District), Lachen and Lachung (North Sikkim). A total of 160 LAB cultures were isolated from these samples and 60 isolates from the human fecal sample in East Sikkim were revived from the previously isolated cultures of the Microbiology Laboratory, Sikkim University. Preliminary characterization was done by observing the morphology and classifying them into rods, short rods, cocci in tetrads and cocci in chains, Gram staining behaviour, KOH string test and catalase test. Then *in vitro* screening for cholesterol lowering of 160 yak milk isolates and 60 human isolates were done in MRS broth supplemented with polyhydroxyethanyl cholesterol sebacate (Sigma, United States), the water soluble cholesterol at a concentration 70-100 μg/ml. On assaying the cholesterol concentration by spectrophotometry, the cholesterol lowering percentage ranged from 10-90%. Those isolates that showed >50% reduction from both the samples (23% in fermented yak milk products and 43% in human fecal), a total of 68 isolates (35 isolates from fermented yak milk and 33 isolates from human feces) were selected. The highest cholesterol removal activity was 90% observed in genus YHC6 followed by 85% in YD5S, 80%, in YW45, YW35, YHC12 and YD12S, 75% in YD15S and 70% in YD7S,YD9S, HS03, HS06, HS08, HS22, HS23, HS29, HS40, HS43, and HS44.

Phenotypic characterisation of 68 isolates to genus level was done according to Axelsson, 2004. Growth at temperatures (10 $^{o}$C, 15 $^{o}$C and 45 $^{o}$C) was observed as it

used mainly to distinguish between some of the lactic acid bacteria. Growth at different salt concentrations like 6.5% NaCl, 10% NaCl and 18% NaCl was also used (Mundt, 1986). Tolerance to acid and/or alkaline conditions may also be useful although not all can grow at the standard test pH of 9.6 and and 4.4. Glucose fermentation was used to classify lactic acid bacteria as the homofermentative, converting glucose almost quantitatively to lactic acid and the heterofermentative, fermenting glucose to lactic acid, ethanol/acetic acid, and $CO_2$ (Sharpe, 1979). The isolates belonged to *Lactobacillus* (46%), *Enterococcus* (28%), *Pediococcus* (20%) and *Lactococcus* (6%) in yak milk products and *Enterococcus* (73%), *Lactococcus* (15%) and *Pediococcus* (12%) in the human fecal isolates.

Acid tolerance, bile tolerance and cell surface hydrophobicity were also determined in order to consider these isolates as probiotic candidate. Tolerance to acid initially at pH 2.5 and then at pH 2 were studied; the degree of viability was isolate specific. In fermented yak milk isolates,12 isolates were most acid tolerant showing more than $10^3$ CFU/ml after incubation of 2 h at pH 2.5, and two isolates showed $10^1$ total CFU/ml. Similarly, 10 fecal isolates showed tolerance at pH 2.5 till 2 h giving $10^2$ CFU/ml. Therefore, 22 isolates (12 fermented yak milk isolates and 10 fecal isolates) were further investigated for pH 2 tolerance for 2 h. All 22 isolates showed tolerance to oxgall, cholic acid and taurocholic acid at 0.5% and 1%. A total of 16 isolates have shown above 40% hydrophobocity and 9 isolates showed more than 75% hydrophobicity.

The isolates were then screened for the presence of BSH enzyme by direct plate assay and classified accordingly into two groups; Group I comprised the BSH positive isolates and Group II comprised the BSH negative isolates. There were 11 isolates of fermented yak milk products and 18 isolates of healthy human faeces belonging to

Group I (11 + 18) and the remaining (14 + 4) showed negative BSH activity in Group II. The BSH positive isolates were assayed for their activity; the BSH producers were capable of showing hydrolysis of both sodium gycocholate (SG) and sodium taurocholate (ST) at varying degrees. The isolates showed more substrate preference towards SG than ST which was in accordance to earlier reports. The highest total activity was observed in *Enterococcus* YY1 (1.30 U/ml) with sodium glychocholate (SG) and *Enterococcus* YHC8 (1.11 U/ml) and *Lactobacillus* YW45 (1.11 U/ml) with sodium taurocholate (ST). Similarly in human fecal isolates, highest value of 1.03U/ml in case of SG was shown by *Enterococcus* HS10 and HS10 *Enterococcus* as 0.93U/ml showed high BSH activity in ST. The LAB isolates, 9 isolates from fermented yak milk samples and 8 isolates from human fecal sample that gave ≥ 0.75U/ml BSH activity was further studied. Bile acid deconjugation was investigated by calculating the amount of cholic acid released with the help of a cholic acid standard curve. The deconjugation or the release of cholic acid ranged from 0.34 to 3.30 mM in yak isolates and from 0.83 to 1.8 mM in human isolates. All isolates were able to hydrolyse both conjugated bile salts at different degrees. In broth containing glycine conjugated bile salt, highest deconjugation was shown by *Lactobacillus* YD11S (3.30mM) from yak milk sample and *Enterococcus* HS29 and *Enterococcus* HS40 (1.81mM) from human fecal sample. Similarly, in sodium taurocholate the highest deconjugation ability was observed in *Enterococcus* HS10 as 1.47 mM and *Lactobacillus* YD11S as 1.98mM. Cholesterol co-precipitation was also studied for the isolates, where the highest precipitation in glycine conjugated bile was shown by *Enterococcus* YD12S, *Lactobacillus* YD9S, *Enterococcus* YY1 and *Enterococcus* HS04 and intaurine conjugated by *Enterococcus* YY1 and *Enterococcus* HS04 respectively.The BSH negative isolates that were grouped as group I were tested for

cholesterol incorporation which ranged from 10 to 72%. The highest degree of cholesterol incorporation was shown by *Enterococcus* YHC12 (72%), followed by *Enterococcus* HS03 (70%), *Lactobacillus* YD15S (68%), *Enterococcus* HS44 (64%) and *Enterococcus* HS12 (62%). Similarly, cholesterol assimilation studies were done and it was observed that the values of cholesterol assimilation ranged from in MRS, cholesterol and 0.3% Ox gall broth. The highest cholesterol assimilation was shown by *Enterococcus* YHC12 (90%) among the yak milk isolates and *Enterococcus* HS03 gave 79% among the human fecal isolates. Similarly sugar fermentation tests were performed using Hi Carbo kit. This was followed by genotypic characterisation of the strains by to 16S rRNA gene sequencing. The strains were identified as *Lactobacillus plantrum* YD5S, *Lactobacillus pentosus* YD8S, *Lactobacillus plantrum* YD9S, *Lactobacillus paraplantrum* YD11S, *Enterococcus faecium* YY1, *Enterococcus thailandicus* HS18, *Enterococcus lactis* YHC20, *Enterococcus durans* HS23, *Enterococcus durans* HS31, *Enterococcus faecium* HS10, *Enterococcus durans* HS03, *Enterococcus durans* HS04, *Enterococcus faecium* HS44 and *Enterococcus faecium* YHC12. The 16S rRNA gene sequences were then submitted to National Center for Biotechnology Information (NCBI) for the accession number. KU601439, KU60140, KU60141, KU60142, KU60143, KU60144, KX274030, KX274031, KX274032, KX274033, KX274034, KX274035, KX354551 and KX387371 were the gene sequence accession number obtained from NCBI. Some isolates were identified as belonging to *Enterococcus* sp. so these strains were tested for antibiotics susceptibility as antibiotic resistance determinants can pass through horizontal gene transfer to the gut pathogens. However, our isolates were found to be completely susceptible to erythromycin, tetracyclin and resistant to chloramphenicol and kanamycin and penicillin.

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# List of Publications
# and Reprints

**List of Publications**

1. Kriti Ghatani and Buddhiman Tamang (2017) Assessment of probiotic characteristics of lactic acid bacteria isolated from fermented yak milk products of Sikkim, India: *Chhurpi, Shyow*, and *Khachu*. Food Biotechnology, 31(3):210–232.

2. Kriti Ghatani and Buddhiman Tamang (2017) Screening of indigenous lactic acid bacteria for cholesterol lowering and additional probiotic attributes. Published in Indian Journal of Agricultural Biochemistry. 30 (1), 85-91. Print ISSN: 0970-6399, Online ISSN: 0974-4479.

3. Kriti Ghatani and Buddhiman Tamang (2016) Indigenous rearing practices of Yak and its multipurpose uses in the Sikkim Himalayas. *IOSR* Journal of Agriculture and Veterinary Science (IOSR-JAVS). 9(3)1:1-8. e-ISSN: 2319-2380, p-ISSN: 2319-2372

4. A manuscript titled "Cholesterol lowering probiotics and its impact on cardiovascular diseases: a review" is communicated for publication as book chapter.

**List of awards received**

1. Young Scientist Award in an International Conference on Nutraceuticals and Functional Foods-The Challenges and Opportunities, organized by Anand Agricultural University, Anand and Indian Society of Agricultural Biochemists Kanpur on 6th – 8th December, 2016 at Anand Agricultural University, Gujarat, India.

2. Best Poster Award securing third position in an International Conference on "Ethnic Fermented Foods and Beverages: Microbiology and Health Benefits" on 20th -21st November, 2015 Organised by Department of Microbiology, Sikkim

University, Gangtok, Sikkim, In Association with SASNET-Fermented Foods at Gangtok.

**List of paper presentations in National and International Conferences/ Seminars**

**Oral Presentation**

1.  Paper presented on "Bio- cultural and Microbial Diversity of Fermented Milk products of Yak in Sikkim Himalayas". National Seminar on Global climate change and its impact on Floral, Faunal and Microbial biodiversity, organised by Dept. of Botany, St. Joseph's College Darjeeling in collaboration with Teesta Torsa Agro Care Welfare Society, Kalimpong held in June 26 to 28, 2015.

2.  Presented paper on "Evaluation of probiotic features in lactic acid bacteria isolated from fermented Yak milk products of Sikkim" Technology Congress, Bardhaman Division, jointly organized by Department of Science and Technology, Govt. of West Bengal and Bankura Christian College, Bankura on 7th – 8th November, 2016 at Bankura Christian College.

3.  Presented paper on "Screening of indigenous lactic acid bacteria for cholesterol lowering and additional probiotic attributes" in International Conference on Nutraceuticals and Functional Foods-The Challenges and Opportunities, organized by Anand Agricultural University, Anand and Indian Society of Agricultural Biochemists Kanpur on 6th – 8th December, 2016 at Anand Agricultural University.

4.  Presented paper on "Bile salt hydrolase activity of indigenous *Enterococcus strains*" in a National Seminar on Frontiers in Cell biology and Microbiology" organized by Dept. of Microbiology, Raiganj University, March 31st 2017.

5. Presented paper on "A study on mechanism of cholesterol lowering by indigenous Lactic acid bacteria" in a National Seminar on Microbial world 2017 " organized by Dept. of Microbiology, University of North Bengal on 4th September. 2017.

6. Presented paper on "Some technological properties of LAB from fermented milk products" in an International conference on Local Issues Global resolutions: Ecology, Environment, Climate and Economy organized by, Raiganj University, 10th-11th January, 2018.

**Poster presentation**

1. International Conference on "Ethnic Fermented Foods and Beverages: Microbiology and Health Benefits" on 20-21 November, 2015 Organised by Department of Microbiology, Sikkim University, Gangtok, Sikkim, India In Association with SASNET-Fermented Foods, titled "Bile salt hydrolase activity of lactic acid bacteria isolated from fermented yak milk products of Sikkim" at Gangtok.

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# Assessment of probiotic characteristics of lactic acid bacteria isolated from fermented yak milk products of Sikkim, India: *Chhurpi, Shyow*, and *Khachu*

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**ABSTRACT**

The present study documents the probiotic attributes of indigenous lactic acid bacteria (LAB) isolated from local fermented Yak milk products namely *Chhurpi, Shyow* and *Khachu* prepared in the northern and eastern region of Sikkim in the Himalayas. Samples were collected aseptically and a total of 170 LAB was isolated and screened for putative probiotic properties like hypocholesteromic effect, acid tolerance, bile tolerance, bile salt hydrolase (BSH) activity and cell surface hydrophobicity. It was observed that 70 LAB isolates showed cholesterol lowering activity, out of which 35 isolates were selected that showed 50% and less cholesterol reducing effect *in vitro*. Acid tolerance test revealed good tolerance of 12 isolates at pH 2.5 and pH 2.0 for up to 2 hours. The tolerance to 0.5% and 1% of three bile salts acid revealed more growth in MRS broth containing taurocholic acid with the isolates revealing good BSH activity leading to bile acid deconjugation. The cell surface hydrophobicity ranged from 20–95%. Furthermore, 16S rRNA gene sequencing revealed *Lactobacillus plantarum* YD5S and YD9S, *L. pentosus* YD8S, *L. paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *E. faecium* YY1 as the best isolates with technological properties. The isolates may serve as potential probiotic candidates with potential for hypocholesteromic benefits in the future.

## Introduction

Fermented yak milk products are a part of livelihood of the ethnic Bhutias and Dukpas living in the Northern and the Eastern part of Sikkim Himalayas (Ghatani and Tamang, 2016). The common traditional fermented yak milk products include *Chhurpi* and *Shyow* (*Dahi* in Nepali), *Thara* or *Khachu* (*Mohi* in Nepali; whey) (Dewan and Tamang, 2007). *Chhurpi* is made of yak milk that is first boiled and whey is added, and then molded into three types: Soft *Chhurpi*, Hard *Chhurpi* and *Dudh Chhurpi*. Soft *Chhurpi* is one of the most common traditional delicacies, consumed as a side dish and chutney being highly palatable and

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replaceable to even the non-vegetarian food in the household of the Sikkimese community. After curdling, it is sieved in a cloth finally giving a soft texture (Fig. 1a). Hard *Chhurpi* is sweet in taste and chewy. After milk is curdled on adding whey it is sieved, packed in a sack and pressed with stones to remove water, then cut into cubical pieces (Fig. 1c). Similarly, *Dudh Chhurpi* is like Hard *Chhurpi*, provided milk is added after cutting it into cubical pieces and dried. It is much more expensive than Hard *Chhurpi* (Fig. 1b).

Butter milk or whey is known as *Khachu* in the local Bhutia language and *Mohi* in Nepali and is obtained as a fermented by-product of *Shyow* or *Dahi*. It is a popular refreshing beverage of the alpine region. In the Bhutia language, curd is known as *Shyow* and *Dahi* in Nepali or the Hindi language. *Shyow* is either naturally fermented yak milk or is prepared from the addition of a starter culture used for fermentation (Ghatani and Tamang, 2016).



Figure 1. The types of fermented yak milk products used in the study. (a) Soft *Chhurpi*; (b) *Dudh Chhurpi*; (c) Hard *Chhurpi* and the different steps in the preparation: (i) the curdled milk pressed with stones to obtain a hard block, (ii) block cut into long pieces, (iii) and then into smaller square pieces.

Overall traditional fermented food has been considered to be enriched with an immense amount of lactic acid bacteria (LAB) of different species. LAB are Gram-positive, catalase negative, non-spore forming, facultative anaerobic cocci or rods, microaerophilic and produce lactic acid when they metabolize carbohydrates (Klein et al., 1998; Axelsson, 2004). There has been growing public attention to the benefits of LAB from traditional foods as part of healthy food options. The present study is an evaluation of probiotic attributes of LAB from the traditionally prepared *Chhurpi, Shyow* and *Thara* or *Khachu*.

An increase in lifestyle diseases based on poor diets has led to demand for healthy food. Many researchers have suggested the therapeutic values of LAB cultures such as probiotics (Lee et al., 1999; Danone, 2001). According to FAO/WHO (2014) probiotic is defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Hill, 2014). The most widely used probiotic bacteria are *Lactobacillus*, a genus of LAB and *Bifidobacterium* (Kailasapathy and Chin, 2000). The health benefits of probiotic bacteria have been recognized in: (1) diseases of the gastrointestinal tract, urogenital and respiratory diseases (Levy, 2000; Reid and Bruce, 2001); (2) diarrheal diseases (Bernet-Camard et al., 1997; Gopal et al., 2001); (3) respiratory tract infections particularly common cold and flu (Hatakka et al., 2001); pneumonia (Taylor and Mitchell, 2007); (4) inflammatory bowel disease including Chron's disease (Gupta et al., 2000); ulcerative colitis, pouchitis; (5) many cancers types like colon cancer, bladder cancer (Ohashi et al., 2002; Kandasamy et al., 2011); (6) hypocholesteromic effect of probiotic lactic acid and bifidobacterial strains (Grill et al., 2000); (7) anti diabetic property (Matsuzaki et al., 2007); and (8) anti allergic potentials in the case of atopic dermatitis in infants (Isolauri et al., 2000).

To be considered an effective probiotic, the strain should be viable, safe, and identified; it should be able to resist the gastric transit, should tolerate bile acid and should have the ability to colonize and adhere in the lining of the gastric mucosa and should have at least one health benefit (Singh et al., 2012). Since there has been no reports on the cholesterol lowering and technological properties of naturally fermented yak milk products of Sikkim, this study investigated the isolation and characterization of LAB from fermented yak milk products to gain insights into potential probiotic properties of the selected LAB isolates. Furthermore, with the advent of molecular techniques for strain identification (Archer and Halami, 2015), 16S rRNA gene sequencing was used for identification of LAB showing the best probiotic potential.

## Materials and methods

### Sample collection

Yak fermented milk products from North and East Sikkim were collected aseptically in sterile sample bottles and transported to the laboratory in ice

packs. Samples were stored at −20°C. Sixteen samples; *Shyow* (*n* = 4), *Khachu* (*n* = 4), hard *Chhurpi* (*n* = 4) and soft *Chhurpi* (*n* = 4) were the fermented milk samples that were analyzed for the probiotic properties of LAB present.

## Microbiological analysis

Ten grams or 10 ml of the sample were homogenized with 90 ml physiological saline for 1 min. Serial dilution of the sample in 0.85% NaCl solution up to $10^{-7}$ dilution was made and plated on de Man, Rogosa and Sharpe (MRS) (HiMedia, Mumbai, India) agar, supplemented with $CaCO_3$ for LAB followed by incubation at 30°C in Anaerobic gas Pack system (HiMedia LE002, Mumbai, India) for 72 h. Colonies were randomly selected (Leisner et al., 1997). A total of 170 pure cultures of the isolates were obtained by streaking on MRS agar without $CaCO_3$. The isolated LAB was preserved in MRS broth using 15% (v/v) glycerol at −80°C. Working cultures were preserved in MRS agar slants at −20°C.

## Preliminary screening

The isolates were subjected to preliminary screening for LAB on the basis of cell morphology, Gram staining and catalase tests (Schillinger and Lucke, 1987).

## In vitro screening for cholesterol lowering

A total of 170 LAB isolates were screened for cholesterol removal or lowering potential by the method described by Liong and Shah (2005). The freshly prepared MRS broth was supplemented with 0.30% oxgall as a bile salt. Water soluble cholesterol (polyoxyethanyl-cholesteryl sebacate; Sigma, Bangalore, India) was filter sterilized and added to the broth at a final concentration of 70–100 μg/ml. The broth was inoculated with each strain at 1% level and incubated anaerobically at 37°C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a colorimetric method using the formula provided in cholesterol testing kit (Erba, Mannheim, Germany). The absorbance of control tube as an uninoculated and inoculated broth was measured at 550 nm. Cholesterol reduction percentage was determined by the following formula (Singh et al., 2012).

$$\text{Cholesterol removal(\%)} = (Control \, (uninoculated \, MRS + Cholesterol)$$
$$- \, inoculated \, MRS + Cholesterol$$
$$\times 100/Control(uninoculated \, MRS + Cholesterol)$$

## Phenotypic characterization to the genus level

The LAB isolates showing cholesterol lowering properties were characterized to the genus level (Axelsson, 2004). These isolates were grouped according to eight characters namely tetrad formation, $CO_2$ production from glucose, growth at temperatures 45°C and 10°C, salt concentrations of 18% and 6.5% NaCl and pH values 4.4 and 9.6 (Schillinger and Lucke, 1987).

## Low pH/acid tolerance

A 1% overnight-grown culture was inoculated in MRS broth supplemented with 0.30% oxgall, the pH of the broth was adjusted to 2.5 with HCl and incubated at 37°C for 2 h (Liong and Shah, 2005). Serial dilutions of the growing cultures were prepared by taking 1 ml broth every 30 min until 2 h; 10-fold serial dilutions were made using peptone water diluents and the tubes were then vortexed for 30 s individually. MRS agar was used for plating and the plates were incubated anaerobically at 37°C for 24 h. The low pH tolerance was determined by comparing the final plate count after 2 h with the initial plate count at 0 h. The experiments were repeated twice. Tolerance of the isolates to pH 2 was also determined by plate count methods as mentioned above.

## Bile salt tolerance

Three different types of bile salts – oxgall, cholic acid and taurocholic acid – were used to study bile tolerance of the isolates (Liong and Shah, 2005) with slight modifications. MRS broths containing 0.5% and 1 % (w/v) of oxgall, cholic acid or taurocholic acid were inoculated with each strain, and incubated at 37°C. MRS broth without bile salt was used as a control according to the method of Gilliland and Walker (1990). Bacterial growth was monitored by measuring the turbidity with a spectrophotometer (Lambda UV-VIS spectrophotometer, Perkin Elmer, Wokingham, UK) at 620 nm at 0 and 8 h, respectively. The pH of the broth cultures was recorded at 0 and after 8 h of incubation. All the experiments were replicated twice.

## Bile salt hydrolase activity

Isolates were evaluated for bile salt hydrolase activity by the plate assay method with slight modification according to a method described by Nguyen et al. (2007). Overnight-grown cultures of the test isolates were streaked on MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA: Sigma) and on MRS plates for comparison. Plates were incubated in an anaerobic jar at 37°C for 72 h, after which the presence of precipitated bile acid around colonies

(opaque halo) or the formation of opaque granular white colonies with a silvery white shine were considered as a positive reaction.

### Cell surface hydrophobicity

The isolates were tested for their ability to adhere to the intestinal lumen by checking the bacterial adhesion to hydrocarbons and results were expressed according to Rosenberg (1984) and Perez et al. (1998). Fresh cultures were grown in MRS broth at 30°C for 24 h and centrifuged at 8,000 $g$ for 5 min. The pellet was washed three times with Ringer solution (Merck, Darmstadt, Germany), and thoroughly mixed in a vortex; 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, Mumbai, India) 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:

$$\text{Hydrophobicity } \% = [A_0 - A/A] \times 100$$

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).

### Molecular characterization

#### DNA extraction

The genomic DNA was extracted using the CTAB method (Cheng and Jiang, 2006).

#### 16S rRNA gene sequencing

Two universal primers 27 F (5′-AGA GTT TGA TCC TGG CTC AG- 3′) and 1492 R (5′AAG GAG GTG ATC CAG CCG CA- 3′) were used for PCR amplification of 16S rRNA region. PCR amplification was done in 20 μl of reaction containing PCR buffer, 1× (KAPA, Cape Town, South Africa); MgCl₂, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase (Sigma, Bangalore, India), 0.05 U; primer, 1 picomol and template DNA, 50 ng in a thermocycler (Biorad, PCR machine). Sterile nuclease free water was used as negative control. The PCR amplification was carried out with DNA denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 48°C for 30 s and elongation at 72°C for 1 min 30 s, followed by a final extension at 72°C for 6 min. The amplicons were purified and sequenced. A volume of 5 μl PCR product was analyzed by electrophoresis in 1.5% Ultrapure™ Agarose (HiMedia, Mumbai, India) gel; at 80 volts for 45 min. Gels were visualized by UV

transillumination. PCR products were purified and sequenced using dideoxy chain termination method. The sequencing reactions were performed using ABI PRISM3100 Genetic Analyzers (Applied Biosystems, Foster City, California, USA) in both directions with universal primers used for amplification.

### Nucleotide sequence accession numbers

The sequences of DNA were aligned. Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in the GenBank database using BLAST. The nucleotide sequences of the 16S rDNA determined in this study were deposited in the NCBI nucleotide sequence databases (https://www.ncbi.nlm.nih.gov).

### Phylogenetic analysis

For phylogenetic analysis, 16S rDNA sequence of the isolates and reference sequence were retrieved from NCBI-GenBank database. The sequences were aligned with Clustal W. The resulting alignment were analyzed with MEGA 6.0 to construct the phylogenetic tree. The phylogenetic tree was deduced with the neighbor-joining (NJ) method (Saitou and Nei, 1987). Sequence divergence among the strain was quantified using Kimura-2-paramater distance model (Kimura, 1980). A total of 1000 bootstrap replication were calculated for evaluation of the tree topology.

## Results

### Isolation and preliminary characterization

A total of 170 colonies of LAB were isolated. All the colonies showed typical circular, low convex, with entire margin and white coloured morphology surrounded by a clear transparent area on MRS agar supplemented with $CaCO_3$. A total of 160 isolates were Gram positive and catalase-negative.

### Cholesterol lowering ability

Out of 160 isolates screened for cholesterol lowering property, the cholesterol lowering percentage ranged from 35–90% in comparison to the control. Maximum cholesterol lowering activity was observed in *Pediococcus* YHC6 (90%), *Lactobacillus* YD5S (85%), *Lactobacillus* YW45 and *Pediococcus* YW35 (80%), *Lactobacillus* YHC12 (80%), *Enterococcus* YD12S (80%), *Lactobacillus* YD15S (75%), *Lactobacillus* YD9S (70%), and *Lactobacillus* YD7S (70%) strains. A total of 35 isolates which showed cholesterol lowering effect above 50% are presented in Figure 2. It shows that these isolates can be potentially used to prevent hypercholesterolemia and therefore selected for further study.
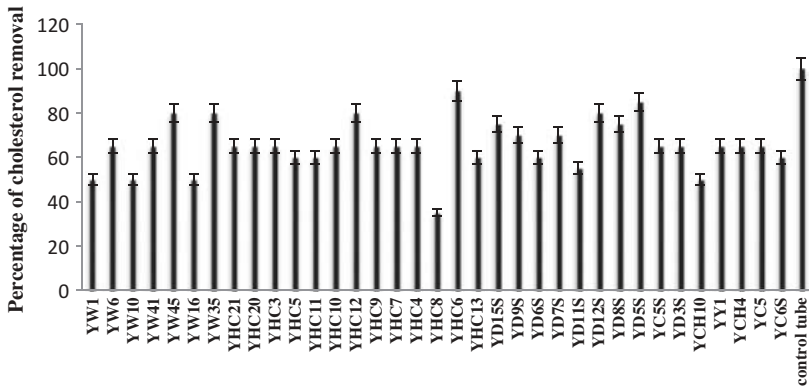
**Figure 2.** Cholesterol lowering activity by 35 selected lactic acid bacteria.

## Physiological and biochemical characteristics

All Gram-positive isolates were divided into rods and cocci bacteria (Table 1). The rod- and coccus-shaped bacteria were segregated further depending on their growth patterns at different temperature, pH, salt concentration, $CO_2$ production from glucose and tetrad formation for classifying into different genera of LAB. The tetrads were placed into *Pediococcus* sp. The rod-shaped, both gas forming and non-gas forming, isolates were placed into *Lactobacillus* sp. The cocci-shaped LAB isolates growing at 6.5% NaCl were grouped into *Enterococcus* whereas those cocci isolates which were not able to grow in 6.5% NaCl were placed into *Lactococcus*.

## Low pH/acid tolerance

The acid tolerance of all 35 LAB isolates with cholesterol lowering properties was studied at a pH of 2.5; the viable counts of pH tolerance by the selective 12 isolates are shown in Table 2. All isolates showed tolerance up to 1 h at pH 2.5 despite the variations in degree of viability. *Pediococcus* YW35, *Enterococcus faecium* YY1, *E. lactis* YHC20, *Enterococcus* YHC12, *Enterococcus* YHC8, *Pediococcus* YHC6, *Enterococcus* YD12S, *Lactobacillus pentosus* YD8S, *L. plantarum* YD5S, *Lactobacillus* YD15S, *L. plantarum* YD9S and *L. paraplantarum* YD11S were most acid-tolerant isolates with more than $10^3$ total CFU/ml after incubation for 2 h at pH 2.5, while *Lactococcus* YCH10 and *Enterococcus* YW1 showed $10^1$ total CFU/ml. The rest of the isolates did not show survival after 2 h. The 12 pH 2.5 tolerant isolates were further screened for tolerance at pH 2. The viable cell counts (log CFU/ml) and survival percentage of selected LAB at pH 2 is presented in Table 3. The isolate *L. plantarum* YD5S and *L. plantarum* YD9S have shown their maximum ability to tolerate the acidic pH for 2 h. For *L. plantarum* YD5S, the counts decreased by 5.5 log cycles and for *L. plantarum* YD9S, the counts

**Table 1.** Phenotypic characters of 35 LAB isolates from fermented milk products of yak.

| Isolate code | Source | Arginine hydrolysis | Tetrad formation | Shape | CO$_2$ production from glucose | Growth in/at | | | | | Genus |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Temperature | NaCl | pH | | | |
| | | | | | | 45°C | 6.5% | 4.4 | 9.6 | | |
| YW1, YY1 | Khachu | + | – | Coccus | – | + | + | 1/1 | + | Enterococcus |
| YW10 | Khachu | + | – | Coccus | – | – | – | + | – | Lactococcus |
| YW6, YW35 | Khachu | – | + | Coccus | – | 1/1 | 1/1 | + | – | Pediococcus |
| YW41, YW45, YW16 | Khachu | 2/1 | – | Rod | 1/2 | 2/1 | + | 1/2 | – | Lactobacillus |
| YHC21, YHC10, YHC9, YHC7, YHC6 | Hard Churpi | + | + | Coccus | – | 2/3 | + | + | – | Pediococcus |
| YHC5, YHC8, YHC20, YHC12, YHC4, YHC13, YCH10, | Hard Churpi | + | – | Coccus | – | + | + | + | + | Enterococcus |
| YCH4 | Hard Churpi | + | – | Coccus | – | – | – | + | – | Lactococcus |
| YHC3, YHC11 | Hard Churpi | 1/1 | – | Rod | – | – | + | + | – | Lactobacillus |
| YC5, YC6S, YC5S | Soft Churpi | 1/2 | – | Rod | – | + | + | + | – | Lactobacillus |
| YD12S | Shyow | + | – | Coccus | – | + | + | + | + | Enterococcus |
| YD8S, YD5S, YD9S, YD11S, YD15S, YD3S, YD6S YD7S | Shyow | 4/4 | – | Rod | – | 1/7 | + | + | – | Lactobacillus |

All isolates showed growth at temperature 10°C. All isolates showed negative result at 18% NaCl. +, all isolates positive; –, all isolates were negative, (.../.../...) number of positive isolates/number of negative isolates.

**Table 2.** Acid tolerance of 35 LAB isolates at pH 2.5.

| Isolates | Viability count at pH 2.5 (log CFU/ml) | | |
|---|---|---|---|
| | 0 h | 1 h | 2 h |
| *Enterococcus* YW1 | 9.50±0.12 | 4.60±0.45 | 1.02±0.23 |
| *Enterococcus faecium* YY1 | 10.13±0.23 | 7.60±0.23 | 5.17±0.12 |
| *Enterococcus lactis* YHC20 | 9.89±0.28 | 7.84±0.11 | 5.89±0.24 |
| *Enterococcus* YHC5 | 8.79±0.15 | 4.24±0.45 | 0 |
| *Enterococcus* YHC12 | 10.40±0.11 | 8.36±0.18 | 4.20±0.10 |
| *Enterococcus* YHC4 | 8±0.12 | 3.11±0.11 | 0 |
| *Enterococcus* YHC8 | 10.05±0.13 | 7.61±0.34 | 3.28±0.12 |
| *Enterococcus* YHC13 | 7.89±0.21 | 3.24±0.18 | 0 |
| *Enterococcus* YD12S | 9.45±0.48 | 6.54±0.39 | 4.15±0.29 |
| *Lactococcus* YW10 | 10.02±0.11 | 3.42±0.40 | 0 |
| *Lactococcus* YCH10 | 10.05±0.11 | 5.05±0.54 | 1±0.12 |
| *Lactococcus* YCH4 | 10.15±0.21 | 4.12±0.32 | 0 |
| *Lactobacillus* YW41 | 9.20±0.12 | 3.20±0.24 | 0 |
| *Lactobacillus* YHC3 | 10.54±0.19 | 5.65±0.11 | 0 |
| *Lactobacillus* YW45 | 8.25±0.34 | 2.01±0.35 | 0 |
| *Lactobacillus* YHC12 | 7.68±0.24 | 3.12±0.59 | 0 |
| *Lactobacillus* YW16 | 10.11±0.54 | 5.02±0.32 | 0 |
| *Lactobacillus* YC5 | 9.85±0.29 | 3.02±0.45 | 0 |
| *Lactobacillus* YC6S | 8.10±0.37 | 4.28±0.49 | 0 |
| *Lactobacillus* YC5S | 8.21±0.12 | 3.10±0.11 | 0 |
| *Lactobacillus pentosus* YD8S | 10.35±0.09 | 7.02±0.27 | 5.63±0.25 |
| *Lactobacillus plantarum* YD5S | 10.13±0.23 | 7.60±0.11 | 5.50±0.14 |
| *Lactobacillus* YD15S | 10.40±0.16 | 8.36±0.19 | 4.20±0.28 |
| *Lactobacillus* YD3S | 8.25±0.18 | 2.89±0.28 | 0 |
| *Lactobacillus plantarum* YD9S | 11.01±0.37 | 8.85±0.16 | 5.50±0.11 |
| *Lactobacillus* YD6S | 8.61±0.11 | 2.50±0.45 | 0 |
| *Lactobacillus* YD7S | 8.79±0.27 | 3.15±0.48 | 0 |
| *Lactobacillus paraplantarum* YD11S | 10.01±0.23 | 7.45±0.11 | 5.05±0.15 |
| *Pediococcus* YHC9 | 8.10±0.27 | 4.25±0.37 | 0 |
| *Pediococcus* YHC7 | 8.25±0.23 | 3.02±0.21 | 0 |
| *Pediococcus* YW35 | 10.62±0.11 | 8.70±0.45 | 4.52±0.11 |
| *Pediococcus* YHC21 | 8.01±0.09 | 2.05±0.23 | 0 |
| *Pediococcus* YHC6 | 9.45±0.15 | 6.05±0.32 | 3.05±0.11 |
| *Pediococcus* YHC10 | 8.01±0.34 | 5.68±0.23 | 0 |
| *Pediococcus* YW6 | 9.23±0.15 | 3.21±0.23 | 0 |

**Table 3.** Acid tolerance of 12 selected LAB isolates at pH 2.

| Isolates | Viability count at pH 2 (log CFU/ml) | | |
|---|---|---|---|
| | 0 h | 1 h | 2 h |
| *Enterococcus* YD12S | 10.5±0.04 | 0 | 0 |
| *Enterococcus* YHC8 | 8.6±0.05 | 0 | 0 |
| *Enterococcus faecium* YY1 | 9.67±0.007 | 3.2±0.01 | 0 |
| *Enterococcus lactis* YHC20 | 9.36±0.004 | 2.45±0.007 | 0 |
| *Lactobacillus paraplantarum* YD11S | 11.17±0.05 | 9±0.05 | 0 |
| *Lactobacillus pentosus* YD8S | 11.29 ±0.001 | 8±0.01 | 0 |
| *Lactobacillus* YD15S | 10.76±0.01 | 8.3±0.05 | 0 |
| *Lactobacillus* YHC12 | 9±0.07 | 0 | 0 |
| *Lactobacillus plantarum* YD5S | 9±0.005 | 6.56±0.007 | 3.5±0.007 |
| *Lactobacillus plantarum* YD9S | 9.028±0.002 | 5.54±0.05 | 3.2±0.01 |
| *Pediococcus* YHC6 | 9.1±0.05 | 0 | 0 |
| *Pediococcus* YW35 | 10.2±0.01 | 0 | 0 |

decreased by 5.83 log cycles, thereby showing greater acid tolerance over entire incubation period of 2 h. For acid-sensitive isolates, the viability decreased slowly for the first hour of incubation followed by rapid decline at the end of the 2-h incubation period.

## Bile tolerance

Oxgall was used as a source of mixed bile, cholic acid as deconjugated bile and taurocholic acid as conjugated bile. MRS broth without bile salt was used as a control in all the experiments. The percentage increase in turbidity/ tolerance and percentage decrease in pH after 8 h exposure was calculated from the initial and final values. The percentage increase in turbidity in MRS broth ranged from 326–1,169%. The effect of 0.5% of cholic acid, taurocholic acid and oxgall is presented in Table 4. The initial pH and the final pH of the media in the presence and in absence of bile salts were monitored. However, the percentage decrease in pH was found not to be related to percentage increase in turbidity. The decrease in pH of the media by different isolates was found to be isolate-dependent. In 0.5% oxgall bile, the percentage increase in turbidity ranged from 43–242%. The highest percentage increase in turbidity was observed in *Enterococcus lactis* YHC20 at 242%, followed by *L. plantarum* YD5S at 210%, *L. pentosus* YD8S at 206% and *L. plantarum* YD9S at 200%. The lowest tolerance was seen in *Lactobacillus* YHC12.

In 0.5% taurocholic acid, the percentage increase in turbidity/tolerance ranged from 341–1,440% and the highest percentage increase in turbidity or tolerance was shown by *Lactobacillus* YD8S at 1440%, *L. plantarum* YD5S at 1,158%, *Pediococcus* YW35 at 1,190% with the lowest tolerance observed in the case of *Pediococcus* YHC6. Similarly, the percentage increase in turbidity or tolerance in 0.5% cholic acid ranged from 36–323%, with the highest percentage increase in turbidity or tolerance seen in *Lactobacillus* YD15S and lowest in *Enterococcus* YHC8 (Table 5).

In most cases, the growth of isolates was found to decrease in 1% bile salt. The isolates had better tolerance in taurocholic acid compared to other two bile salts. In 1% oxgall bile, the percentage increase in turbidity or tolerance ranged from 13–187% with the highest tolerance in *Lactobacillus* YD15S and lowest in *Enterococcus* YHC8. The percentage increase in turbidity or tolerance was in the range of 275–1,356% in 1% taurocholic acid. The highest tolerance was shown by *Lactobacillus* YD8S at 1356%, followed by *L. plantarum* YD5S at 1,250%, *Enterococcus* as YD12S at 820% and *L. paraplantarum* YD11S at 800%. The lowest tolerance was shown by *E. lactis* YHC20.

In 1% cholic acid, the range of % increase in turbidity or tolerance was 25–246%. The highest was shown by YD12S *Enterococcus* and lowest by *L. paraplantarum* YD11S. The isolates showed more tolerance to 1%

**Table 4.** Bile tolerance of lactic acid bacteria in different bile media 0.5%.

| | Growth media | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MRS broth | | MRS broth + 0.5% Ox bile | | MRS broth + 0.5 taurocholic acid | | MRS broth + 0.5% cholic acid | |
| Isolates | % Increase in turbidity/tolerance | % Decrease in pH | % Increase in turbidity/tolerance | % Decrease in pH | % Increase in turbidity/tolerance | % Decrease in pH | % Increase in turbidity/tolerance | % Decrease in pH |
| *Enterococcus* YD12S | 807 | 14.15 | 170 | 3.59 | 791 | 15.29 | 92 | 3.71 |
| *Enterococcus* YHC8 | 782 | 16.61 | 47 | 1.35 | 757 | 16.04 | 36 | 2.10 |
| *Enterococcus faecium* YY1 | 778 | 12.85 | 94 | 2.99 | 892 | 15.74 | 107 | 2.82 |
| *Enterococcus lactis* YHC20 | 735 | 16.15 | 242 | 3.60 | 788 | 17.39 | 317 | 10.68 |
| *Lactobacillus paraplantarum* YD11S | 814 | 13.84 | 156 | 2.99 | 960 | 15.44 | 90 | 5.79 |
| *Lactobacillus pentosus* YD8S | 1150 | 14.76 | 206 | 4.19 | 1440 | 16.5 | 110 | 7.86 |
| *Lactobacillus* YD15S | 326 | 13.23 | 105 | 1.05 | 423 | 7.04 | 263 | 9.50 |
| *Lactobacillus* YHC12 | 479 | 10.62 | 43 | 1.05 | 473 | 8.7 | 78 | 0.15 |
| *Lactobacillus plantarum* YD5S | 1169 | 18.15 | 210 | 7.50 | 1158 | 13.34 | 146 | 3.56 |
| *Lactobacillus plantarum* YD9S | 607 | 13.85 | 200 | 5.70 | 662 | 10.04 | 275 | 3.86 |
| *Pediococcus* YHC6 | 395 | 12.62 | 136 | 2.54 | 341 | 8.7 | 323 | 10.39 |
| *Pediococcus* YW35 | 993 | 14.92 | 125 | 3.30 | 1190 | 15.14 | 145 | 3.86 |

Table 5. Bile tolerance of lactic acid bacteria in different bile media 1%.

| Isolates | Growth media | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MRS broth | | MRS broth + 1% Ox bile | | MRS broth + 1% taurocholic acid | | MRS broth + 1% cholic acid | |
| | % Increase in turbidity/tolerance | % Decrease in pH | % Increase in turbidity/tolerance | % Decrease in pH | % Increase in turbidity/tolerance | % Decrease in pH | % Increase in turbidity/tolerance | % Decrease in pH |
| *Enterococcus* YD12S | 807 | 14.15 | 44 | 2.39 | 820 | 14.09 | 69 | 5.04 |
| *Enterococcus* YHC8 | 782 | 16.61 | 13 | 3.14 | 757 | 14.84 | 71 | 4.89 |
| *Enterococcus faecium* YY1 | 778 | 12.85 | 94 | 2.54 | 515 | 16.34 | 83 | 1.18 |
| *Enterococcus lactis* YHC20 | 735 | 16.15 | 150 | 2.39 | 275 | 10.04 | 233 | 4.59 |
| *Lactobacillus paraplantarum* YD11S | 814 | 13.84 | 60 | 1.94 | 800 | 14.50 | 33 | 3.56 |
| *Lactobacillus pentosus* YD8S | 1150 | 14.76 | 187 | 5.39 | 1356 | 18.14 | 120 | 5.78 |
| *Lactobacillus* YD15S | 326 | 13.23 | 90 | 2.55 | 233 | 10.04 | 159 | 8.01 |
| *Lactobacillus* YHC12 | 479 | 10.62 | 33 | 2.40 | 436 | 9.29 | 25 | 0.59 |
| *Lactobacillus plantarum* YD5S | 1169 | 18.15 | 181 | 5.39 | 971 | 17.54 | 200 | 3.44 |
| *Lactobacillus plantarum* YD9S | 607 | 13.85 | 105 | 2.24 | 533 | 16.04 | 246 | 6.37 |
| *Pediococcus* YHC6 | 395 | 12.62 | 32 | 1.04 | 306 | 9.29 | 100 | 2.07 |
| *Pediococcus* YW35 | 993 | 14.92 | 113 | 5.55 | 991 | 14.84 | 91 | 0.44 |

taurocholic acid like in 0.5% taurocholic acid. The comparison of % increase in turbidity or tolerance is presented in Table 6.

## Bile salt hydrolase activity of isolates

The isolates were grown at 0.5% bile salt concentration to understand the ability of bile salt hydrolysis. The presence of opaque granular white colonies with white shine was considered as a positive reaction. Depending on confluent the precipitation each isolate was given codes and denoted by '+++' for heavy, '++' for intermediate, '+' for less and no precipitation denoted by '_' (Table 7). Among 12 isolates screened by direct plate assay, eight isolates revealed heavy precipitation of bile acid (Fig. 3).

**Table 6.** Percentage increase in turbidity and tolerance in MRS broth, MRS broth + 0.5% bile salt, and MRS broth + 1% bile salt by LAB.

| | | Growth media | | | | | |
|---|---|---|---|---|---|---|---|
| | | % Increase in turbidity/ tolerance in MRS broth + 0.5% bile salt | | | % Increase in turbidity/ tolerance in MRS broth + 1% bile salt | | |
| Isolates | % Increase in turbidity/tolerance in MRS broth | Ox bile | Taurocholic acid | Cholic acid | Ox bile | Taurocholic acid | Cholic acid |
| *Enterococcus* YD12S | 807 | 170 | 791 | 92 | 44 | 820 | 69 |
| *Enterococcus* YHC8 | 782 | 47 | 757 | 36 | 13 | 757 | 71 |
| *Enterococcus faecium* YY1 | 778 | 94 | 892 | 107 | 94 | 515 | 83 |
| *Enterococcus lactis* YHC20 | 735 | 242 | 788 | 317 | 150 | 275 | 233 |
| *Lactobacillus paraplantarum*YD11S | 814 | 156 | 960 | 90 | 60 | 800 | 33 |
| *Lactobacillus pentosus* YD8S | 1150 | 206 | 1440 | 110 | 187 | 1356 | 120 |
| *Lactobacillus* YD15S | 326 | 105 | 423 | 263 | 90 | 233 | 159 |
| *Lactobacillus* YHC12 | 479 | 43 | 473 | 78 | 33 | 436 | 25 |
| *Lactobacillus plantarum* YD5S | 1169 | 210 | 1158 | 146 | 181 | 971 | 200 |
| *Lactobacillus plantarum* YD9S | 607 | 200 | 662 | 275 | 105 | 533 | 246 |
| *Pediococcus* YHC6 | 395 | 136 | 341 | 323 | 32 | 306 | 100 |
| *Pediococcus* YW35 | 993 | 125 | 1190 | 145 | 113 | 991 | 91 |

**Table 7.** Bile salt hydrolase (BSH) activity of 12 LAB isolates.

| Isolates | BSH activity |
|---|---|
| *Enterococcus* YD12S | + |
| *Enterococcus* YHC8 | +++ |
| *Enterococcus faecium* YY1 | +++ |
| *Enterococcus lactis* YHC20 | +++ |
| *Lactobacillus paraplantarum* YD11S | +++ |
| *Lactobacillus pentosus* YD8S | +++ |
| *Lactobacillus* YD15S | – |
| *Lactobacillus* YHC12 | – |
| *Lactobacillus plantarum* YD5S | +++ |
| *Lactobacillus plantarum* YD9S | +++ |
| *Pediococcus* YHC6 | – |
| *Pediococcus* YW35 | – |

Note: Maximum precipitation is denoted by '+++' and no precipitation is denoted by '–'.

*Enterococcus* YD12S ("+")



*Lactobacillus pentosus* YD8S ("+++")

**Figure 3.** BSH activity of lactic acid bacteria isolates showing less and more precipitation.
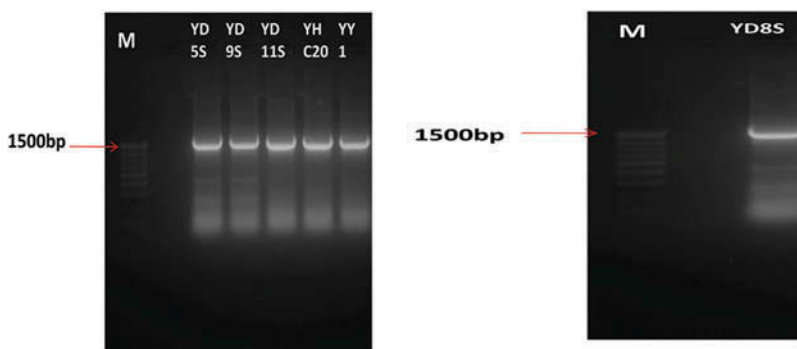


**Figure 4.** Gel photograph of 16S rRNA PCR of selected lactic acid bacterial isolates.

## *Cell surface hydrophobicity*

The cell surface hydrophobicity values ranged from 20.50–94.80% (Table 8). Maximum hydrophobicity was shown by *L. pentosus* YD8S (94.80%)

**Table 8.** Cell surface hydrophobicity of the LAB isolates.

| Isolates | Cell surface hydrophobicity % |
|---|---|
| *Enterococcus* YD12S | 58.13 |
| *Enterococcus* YHC8 | 79.59 |
| *Enterococcus faecium* YY1 | 54.99 |
| *Enterococcus lactis* YHC20 | 85.18 |
| *Lactobacillus paraplantarum* YD11S | 75.01 |
| *Lactobacillus pentosus* YD8S | 94.8 |
| *Lactobacillus* YD15S | 20.5 |
| *Lactobacillus* YHC12 | 53.45 |
| *Lactobacillus plantarum* YD5S | 71.45 |
| *Lactobacillus plantarum* YD9S | 66.94 |
| *Pediococcus* YHC6 | 62.38 |
| *Pediococcus* YW35 | 77.68 |

followed by *E. lactis* YHC20 (85.18%), *Enterococcus* YHC8 (79.59%), *Pediococcus* YW35 (77.68%), *L. paraplantarum* YD11S (75.01%), *L. plantarum* YD5S (71.45%), *L. plantarum* YD9S (66.94%) and *Pediococcus* YHC6 (62.38%). These isolates may be considered as a potential probiotic culture from the adherence test.

## Molecular characterization

Molecular identification of the isolates was performed for six isolates depending on the results of cholesterol lowering, acid and bile tolerance, BSH activity and cell surface hydrophobicity by amplifying and sequencing the 16S rRNA gene sequences and comparing the results to the database of known 16S rRNA sequences by BLAST. During BLAST, three isolates namely *Lactobacillus* YD8S, *Lactobacillus* YD11S and *Lactobacillus* YD9S showed 100% coverage to three species of *Lactobacillus*, i.e. *L. plantarum, L. paraplantarum* and *L. pentosus.* Further identification of the isolates by sugar fermentation tests to differentiate between *L. plantarum, L. paraplantarum* and *L. pentosus* were performed (Curk et al., 1996). LAB isolates were grown in MRS broth supplemented with glycerol, D-xylose, L-rhamnose, D-Arabitol and D-melizitose. *Lactobacillus* YD9S gave a positive result for D-Arabitol and D-melizitose, *Lactobacillus* YD11S gave a positive result only for D-melizitose and *Lactobacillus* YD8S gave positive results for glycerol and D-xylose, respectively. After verification with sugar tests YD9S was identified as *L. plantarum*, YD11S as *L. paraplantarum* and YD8S as *L. pentosus*. The selected six isolates were identified as: *L. plantarum* YD9S and YD5S, *L. paraplantarum* YD11S, *L. pentosus* YD8S, *Enterococcus lactis* YY1 and *E. faecium* YHC20.

YD8S *L. pentosus* (1,452 bp), YD5S *L. plantarum* (1,480 bp) YD11S *L. paraplantarum* (1,472 bp), YD9S *L. plantarum* (1,012 bp), YY1 *Enterococcus faecium* (769 bp), YHC20 *E. lactis* (1,474 bp) were provided KU601439, KU601440, KU601441, KU601442, KU601443 and KU601444 accession numbers, respectively. A phylogenetic tree was constructed with the neighbor
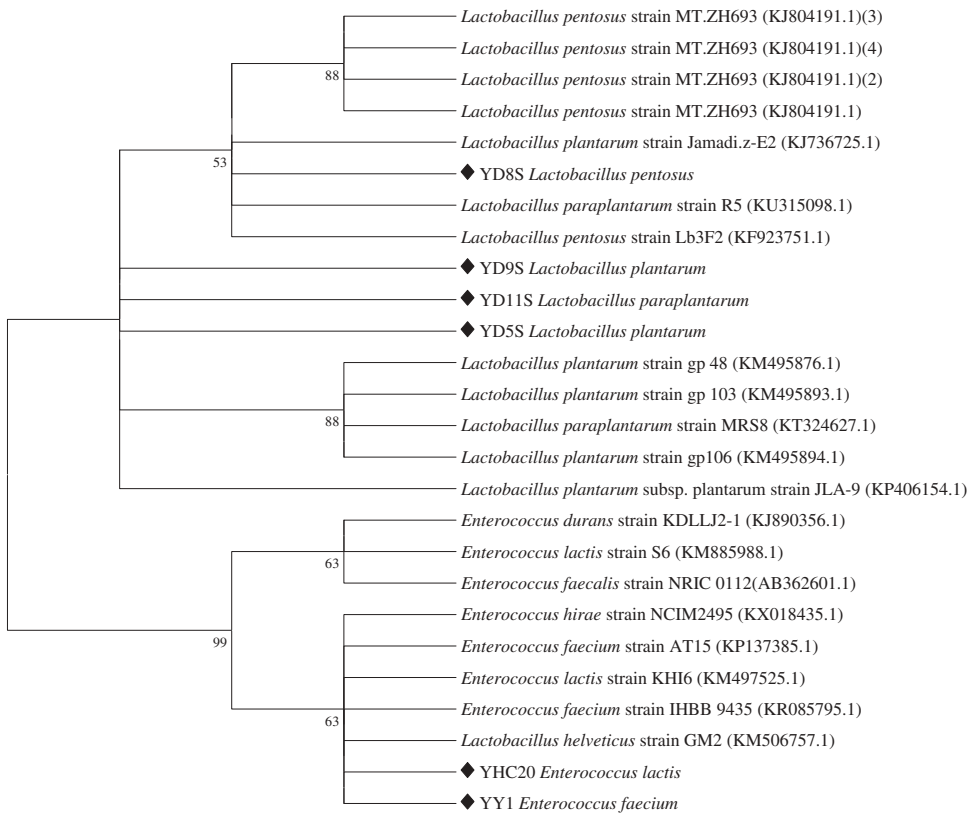
**Figure 5.** The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.15683622 was obtained. The evolutionary distances were computed using the Kimura 2-parameter method.

joining method based on the evolutionary distance calculated from 1,000 replicates shown in Figure 5. Out of two strains, four strains showed 99% similarity to *Enterococcus*.

## Discussion

In the present study, LAB isolated from fermented milk products of Yak were assessed for *in vitro* probiotic attributes. Out of 35 isolates, six isolates showing good probiotic properties with cholesterol-lowering effects and were identified as *L. plantarum* YD5S and YD9S, *L. pentosus* YD8S, *L. paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *E. faecium* YY1.

The most important characteristics that probiotic microorganisms should possess are tolerance to acid, tolerance to bile and cell surface hydrophobicity. For a strain to be a probiotic, it should have at least one health benefit. The novel strains having anti-hypercholesterolaemic properties were obtained from *Chhurpi, Shyow* and *Thara* or *Khachu*. The cholesterol-lowering percentage by our isolates ranged from 15–80% from broth media

containing water soluble cholesterol. The isolates which showed ≥ 50% cholesterol-lowering ability were studied for tolerance to acid, bile salt and cell surface hydrophobic nature to check their ability to survive in the gastrointestinal tract to exert a beneficial effect on the host. The percentage of cholesterol lowering of 0.4–47% in the media was earlier reported by Pereira and Gibson (2002). High cholesterol removal by the isolates might indicate the importance to the bacterial cell growth. The time from the consumption of probiotic food to the discharge in the stomach has been estimated as 90 min (Berada et al., 1991). The LAB selected for probiotic property must be able to resist the high acidity that exists in the stomach in order to colonize and exert a positive effect. The pH of gastric juice secreted in the stomach is about 2 and many microorganisms are destroyed at this and lower pH. So, the tolerance to acidic conditions is a very important criterion for selection of probiotic bacteria (Huang and Adams, 2004). The survival in the gastrointestinal tract is an important feature of a probiotic microorganism and to remain viable to produce beneficial effects. The pH between 1.5 and 3.0 in stomach (Corzo and Gilland, 1999) and bile acid presence in the upper intestine are the stresses involved. The pH 2.0 was regarded as a strong discriminative pH for the selection of high acid-tolerant strains (Turchi et al., 2013). The isolates were then tested for their ability to tolerate a higher pH of 2.5 for 2 h. At pH 2.5, some isolates showed reduced viability that were similar to the results obtained in *L. casei* (Nagata et al., 2009). The decrease of viability of LAB at low pH was also reported by Raghavendra et al. (2010). All the isolates showed different tolerance at varying pH. The isolates were studied at pH of 2 initially for 2 h; this was similar to *Lactobacillus acidophilus* ATCC 4357, *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 290 that survived best at pH 2 as in studies conducted by Liong and Shah (2005). Tolerance to bile salts is another obstacle that must be overcome by probiotic bacteria in the small intestine. Bile salts are the main components of bile, which are capable of disrupting the structure of cell membranes, thus being toxic to living cells (Begley et al., 2006). Growth was observed in 0.3% of three different bile salts sources taurocholic acid (conjugated bile), oxgall (mixed bile) and cholic acid (deconjugated bile) as studied by Liong and Shah (2005). However, in our studies two different concentrations of bile salts 0.5% and 1% were used as there had been reports of the concentration of bile salts in the small intestine ranging from approximately 0.2–2% (wt/vol), depending upon the individual and the type and amount of food ingested (Dawson, 1998). However, the appropriate physiological concentration of human bile ranges from 0.1–0.3% (Dunne et al., 2001) and 0.5% (Mathara et al., 2008) and the residing times are proposed to be 4 h (Mishra and Prasad, 2005). It was reported that 2% bile salt concentration is detectable only at the first hour of digestion and it decreases gradually to 0.3% during prolonged digestion time (Noriega et al.,

2004). The ability to tolerate bile salts in MRS broths with 1.0, 0.5 and 0.3% was studied for 42 *Lactobacillus plantarum* strains isolated from different Italian food sources (milk, cheese, fermented meat products) by Turchi et al. (2013). Our isolates had the ability to grow in MRS broth supplemented with taurocholic acid and this may be because they produce bile salt hydrolase enzymes that deconjugate the conjugated bile (Moser and Savage, 2001; Taranto et al., 2006). The BSH activity of the isolates revealed the ability to hydrolyze bile salt causing precipitation, thereby increasing the survival of bacteria under bile salt toxicity (Pisano et al., 2008).

In some bacteria, the resistance towards conjugated bile may be because of greater solubility and detergent activity (Liong and Shah, 2005). Three of the *Lactobacillus* strains obtained from infant faeces and pickled vegetables showed greater viability reduction at 1% bile salt when compared with 0.3% bile salt (Wang et al., 2010). The bacterial adhesion to hydrocarbons to study the ability of the bacteria to adhere to the surface of host cells determines the colonization capability of bacteria, which is a crucial step in the establishment of probiotics (Orlowaski and Bielecka, 2006). In determining cell surface hydrophobicity which is considered as one of the major features of probiotic bacteria, hydrophobicity more than 40% was considered as hydrophobic (Boris et al., 1998). However, maximum isolates have shown a hydrophobicity percentage of more than 40%. The high values of hydrophobicity relate a greater ability of bacteria to adhere to the epithelial cells as indicated by Rosenberg (1984). In another study by Tamang et al. (2009), hydrophobicity greater than 70% was found in *L. brevis* strains MeN7 (BFE942) (94.5%), MeR6 (BFE938) (91.5%), SL: B7 (BFE2889) (84%), KG: B2 (BFE952) (81%), MeTR (BFE941) (72%) and *L. plantarum* strains MeL2 (BFE934) (94%), MeL3 (BFE944) (75%). The results obtained for hydrophobicity in n-hexadecane ranged from 37.80–85.67, 21.06–88.00 and 76.33%, respectively, for *L. paracasei, L. plantarum*, and *L. brevis* (Jamaly et al., 2011) and in *L. acidophilus* ranged from 38.1–67.8% (Vindderola and Reinheimer, 2003).

An increase in cholesterol level above the normal in blood results in increased risk of cardiovascular diseases. LAB isolates were screened for cholesterol-lowering ability and showed maximum lowering of 85% by *L. plantarum* YD5S, 70% by *L. plantarum* YD9S, 70% by *L. pentosus* YD8S, 55% by *L. paraplantarum* YD11S, 65% by *Enterococcus lactis* YHC20 and *E. faecium* YY1. Although *Pediococcus* YW35 and YHC6 strains revealed good cholesterol-lowering ability, bile tolerance and cell surface hydrophobicity, the two strains were BSH-negative and non-acid tolerant at pH 2 for up to 1 h, hence they were not identified as suitable. The observed hypocholesteromic effect in test tube studies among these indigenous food strains of LAB could be due to the bile salt hydrolase activity that results in deconjugation of bile salts. This may benefit health by cholesterol not being absorbed in the body and may allow new bile to start to synthesize from cholesterol and therefore could reduce the total concentration of cholesterol in the vascular

system. *Lactobacillus plantarum* YD5S, *L. plantarum* YD9S, *L. pentosus* YD8S, *L. paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *E. faecium* YY1 exhibited hypocholesteromic effects and probiotic attributes that could be targeted to prevent the risk of cardiovascular diseases.

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# Screening of Indigenous Lactic Acid Bacteria for Cholesterol Lowering and Additional Probiotic Attributes[#]

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The study was aimed to evaluate the cholesterol lowering property and probiotic attributes of lactic acid bacteria (LAB) isolated from indigenous fermented milk products of Yak from the eastern region of Sikkim Himalayas. A total of 7 indigenous LAB were screened for putative probiotic features namely hypocholesterolaemic effect, bile salt hydrolase activity, acid tolerance and cell surface hydrophobocity. The possible mechanisms of cholesterol lowering like bile salt hydrolase (BSH) enzyme activity, bile acid deconjugation, cholesterol co-precipitation and cholesterol incorporation were studied. Isolates were subjected to direct plate assay in MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt for the presence of bile salt hydrolase (BSH) enzyme. Screening revealed *Lactobacillus paraplantarum* YD11S and *Lactobacillus plantarum* YD5S showing bile acid deconjugation while co-precipitation was observed in YY1 *Enterococcus lactis* and *Lactobacillus pentosus* YD8S as the cholesterol lowering mechanism. *Lactobacillus plantarum* YD9S showed good bile acid deconjugation and co-precipation. *Enterococcus faecium* YHC12 was BSH-ve and showed cellular incorporation to be its mechanism of cholesterol lowering.

**Key words:** Probiotic, indigenous, bile salt hydrolase enzyme, cholesterol lowering mechanism

Cardiovascular diseases have been the most important cause of morbidity and mortality in many industrialised countries in the world. Due to urbanization and decreased use of food grains, vegetables and stressful life (1), the risk due to cardiovascular disease; stroke and heart failure are increasing at an alarming rate. Lipid cholesterol accumulates in the arteries of the heart causing plaque formation thereby blocking the flow of blood from the heart ventricles and to the rest part of the body. Several studies and clinical trials have shown a link between cholesterol elevation and cardiovascular diseases. Probiotics and prebiotics are known to have hypocholesterolaemic effect. However there have been many definitions of probiotics literally meaning 'for life' (2). However, according to FAO/WHO definition, probiotics have been defined as "live microorganisms when administered in adequate amounts confer a health benefit on the host" (3).

There have been reports of lactic acid bacteria in dairy products that reduce cholesterol level (4, 5). In several *in vitro* studies a number of mechanisms have been proposed for the cholesterol-lowering action of probiotic bacteria (6-9) although the exact mechanism of cholesterol lowering is poorly understood and the hypotheses for mechanism need to be confirmed in animal and human studies. The mechanisms for cholesterol lowering by probiotics include, binding of cholesterol to the cellular membrane (9), assimilation of cholesterol by the growing cells (10-12), cholesterol incorporation into the cellular membrane, bile salt hydrolase (BSH) or products containing them have been suggested to lower cholesterol levels through interaction with host metabolism of bile salt (13), deconjugation of bile salts by the action of bile salt hydrolase enzyme (14) and co-precipitation of cholesterol with deconjugated bile (15,11).

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## Materials and Methods

***Bacterial strain and culture conditions:*** Seven native *Lactobacillus* and *Enterococcus* isolated from fermented milk products of Yak namely hard *Chhurpi* and *Shyow* (*Dahi* in Nepali), *Thara* or *Khachu* (*Mohi* in Nepali; whey) were used for the study. The bacterial cultures were identified by biochemical, 16s rRNA gene sequencing as well as phylogenetic analysis. All the strains were maintained at -20 ° C in 20% glycerol (v/v). The isolates were grown in de Man, Rogosa, Sharpe (MRS) broth (HiMedia, Mumbai, India). Prior to analysis, the cultures were propagated in broth mediums twice. YD5S *Lactobacillus plantarum* KU601439, YD8S *Lactobacillus pentosus* KU601440, YD9S *Lactobacillus plantarum* KU601441, YD11S *Lactobacillus paraplantarum* KU601442, YHC20 *Enterococcus lactis* KU601443, YY1 *Enterococcus faecium* KU601444 and YHC12 *Enterococcus faecium* KX387371 were the strains used, the 16s rRNA sequences of which were deposited to NCBI and accession numbers were obtained.

***Bile salt hydrolase activity:*** The bile salt hydrolase activity of the strains was determined by the direct plate assay method. MRS agar supplemented with 0.5% (w/v) taurodeoxycholic acid of sodium salt (TDCA: Sigma) and only MRS agar was used for comparison plates for each (16). Plates were incubated in anaerobic jar at 37° C for 72 h, after which the presence of precipitated bile acid around colonies (opaque halo) or the formation of opaque granular white colonies with a silvery white shine were considered as a positive reactions.

### Screening for cholesterol lowering property: LAB isolates were screened for cholesterol removal or lowering potential by the method described by Liong and Shah (2005) (14). The freshly prepared MRS broth was supplemented with 0.30% oxgall as a bile salt. Water soluble cholesterol (polyoxyethanyl-cholesterylsebacate; Sigma) was filter sterilized and added to the broth at a final concentration of 70-100 ìg/ml. The broth is then inoculated with each strain at 1% level and incubated anaerobically at 37°C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth is determined using a colorimetric method with cholesterol testing kit (Erba, Manninhem).

***BSH assay:*** BSH activity was measured by determining the amount of amino acids liberated from conjugated bile salts by lactobacilli strains as described by Tanaka, Hashiba, Kok, and Mierau (2000) (17), with several modifications. Cells were grown in MRS broth for 20 h after which it was centrifuged at 10 000 g at 4°C for 10 min. The cell pellet was washed twice before suspension into 10mL of 0.1M phosphate buffer (pH 7.0). The cell concentration was adjusted to an OD value of 1 unit at 600 nm. 5ml of the cell suspension was sonicated for 3 min with constant cooling in ice, followed by centrifugation at 10 000 g at 4°C for 10 min. To 0.1mL of appropriately diluted supernatant obtained, 1.8ml of 0.1M sodium phosphate buffer (pH 6) and 0.1ml of conjugated bile salt was added. Conjugated bile used was 6mM sodium glycocholate and 6mM sodium taurocholate. The mixture will be incubated at 37°C for 30 min. Enzymatic reaction was terminated by adding 0.5ml of trichloroacetic acid (15% wt/vol) to 0.5mL of sample. The mixture was centrifuged and 0.2ml of supernatant obtained was added to 1ml of distilled water and 1ml of ninhydrin reagent (0.5mL of 1% ninhydrin in 0.5M citrate buffer pH 5.5, 1.2mL of 30% glycerol, 0.2mL of 0.5M citrate buffer pH 5.5). The preparation was vortexed and then boiled for 14 min. After subsequent cooling, the absorbance at 570nm was determined using glycine or taurine as standards. One unit of BSH activity was defined as the amount of enzyme that liberated 1mmol of amino acid from substrate per min. Protein concentrations were determined by the Lowry method (18) with bovine serum albumin as the standard. All experiments were repeated thrice.

***Deconjugation of sodium glycocholate and sodium taurocholate:*** 10ml volumes of MRS broth were supplemented with 6mM sodium glycocholate, 6mM sodium taurocholate, respectively. Each strain was inoculated at the 1% level and incubated anaerobically at 37°C for 20 h. 10mL culture of each organism after the incubation period was adjusted to pH 7.0 with NaOH (1 N). The cells were centrifuged at 10 000 g (Remi Cooling Centrifuge Model C 30- BL, India) at 4°C for 10 min. Supernatant obtained was adjusted to pH 1.0 with HCl (10 N). 1ml of the supernatant was added with 2mL of ethyl acetate and the mixture was vortexed for 1 min.

2ml of the ethyl acetate layer was transferred into a glass tube and evaporated under nitrogen at 60° C. The residue was immediately dissolved in 1mL of NaOH (0.01 N). After complete mixing, 1mL of furfuraldehyde (1%) and 1mL of $H_2SO_4$ (16 N) were added, and the mixture is vortexed for 1 min before heating at 65°C in a water bath for 10 min. After cooling, 2mL of glacial acetic acid was added and the mixture was vortexed for 1 min. Absorbance was read at 660 nm (Lamda 25, Perkin Elmer, UV Vis, spectrophotmeter). The amount of cholic acid released was determined using cholic acid standard. All experiments were replicated thrice (19).

**Co-precipitation of cholesterol with deconjugated bile:** Freshly prepared sterile MRS broth was supplemented with 6mM sodium glycocholate and 6mM sodium taurocholate (11). Cholesterol was filter sterilized and added to the broth at a final concentration of 70–100 µg/ml. The broth was inoculated at the 1% level with each strain and incubated anaerobically at 37°C for 20h. The water-soluble cholesterol had a solubility of 60mg/ml and contained 30% cholesterol. Thus, initial cholesterol concentration varies between 70 and 100 µg/ml and is quantified separately for every batch. After the incubation period, cells were centrifuged (10,000 g, 4°C, 10 min) and the remaining cholesterol concentration in the broth was determined using cholesterol testing kit (Erba, Manninhem).

**Cholesterol incorporation:** The incorporation of cholesterol into the cell membrane of the bacterial strains was studied according to the work described by Noh *et al.* (9). The MRS-THIO broth was prepared by supplementing lactobacilli MRS broth with 0.2% sodium thioglycolate. The broth was further supplemented, when desired, with 0.004 M sodium taurocholate or 0.3% oxgall. The broth media was then autoclaved for 15 min at 121°C. The measurement of cholesterol assimilation was done as follows; one milliliter of cholesterol-phosphatidylcholine micelles was prepared according to Razin et al. (20) and added to the tubes containing 9 ml of MRS-THIO broth. Cholesterol testing kit (Erba, Maneinham) was used to determine the amount of cholesterol in the spent broth and in the uninoculated control.

**Low pH or acid tolerance:** To screen for acid tolerance 1% overnight grown culture was inoculated in MRS broth supplemented with 0.30% oxgall. The pH of the broth was adjusted to 2.5 with HCl and incubated at 37°C for 2 h (14). Serial dilution of the growing cultures were prepared by taking 1ml broth every 30 min till 2 h, 10-fold serial dilutions were made using peptone water diluents, the tubes were vortexed for 30 s individually. MRS agar was used for plating and the plates were incubated anaerobically at 37 °C for 24 h in an anaerobic condition. The low pH tolerance was determined by comparing the final plate count after 2 h with the initial plate count at 0 h. The experiments were repeated twice.

**Cell surface hydrophobocity:** The isolates were tested for their ability to adhere to the intestinal lumen by checking the bacterial adhesion to hydrocarbons and results were expressed according to Rosenberg (1984) (21) and Perez et al. (1998) (22). Fresh cultures were grown in MRS broth at 30°C for 24 h and centrifuged at 8,000g for 5 min. The pellet was washed three times with Ringer solution (Merck, Germany), and thoroughly mixed in a vortex. 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, 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 % = $[A_0\text{-}A/A]$ x 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 (23, 24).

## Results and Discussion

**Bile salt hydrolase activity:** On screening the isolates for the presence of bile salt hydrolase enzyme (BSH), YD5S *Lactobacillus plantarum*, YD8S *Lactobacillus pentosus*, YD9S *Lactobacillus plantarum*, YD11S *Lactobacillus paraplantarum*, YHC20 *Enterococcus lactis* and YY1 *Enterococcus faecium* showed positive

BSH activity while YHC12 *Enterococcus faecium* gave a negative result (Table 1). Depending on how confluent the precipitation was each isolate was given codes as heavy denoted by "+++", intermediate denoted by "++", less denoted by "+" and none denoted by "_". The formation of opaque white colonies showed the ability of the isolates to produce BSH enzyme, an enzyme that is able to deconjugate bile salts to simple bile acid. The presence of BSH enzyme is an important mechanism for the reduction of blood cholesterol (25). Those isolates which gave positive result were groups as BSH positive strains and studied further for other BSH related quantification. Only one isolate was grouped as BSH negative.

**Table 1:** BSH activity of LAB strains

| Isolate | BSH activity |
|---|---|
| *Lactobacillus plantrum* YD5S (KU601439) | +++ |
| *Lactobacillus pentosus* YD8S (KU601440) | +++ |
| *Lactobacillus plantrum* YD9S (KU601441) | +++ |
| *Lactobacillus paraplantrum* YD11S (KU601442) | +++ |
| *Enterococcus faecium* YY1 (KU601444) | +++ |
| *Enterococcus lactis* YHC20 (KU601443) | +++ |
| *Enterococcus faecium* YHC12 (KX387371) | - |

**Cholesterol removal:** The cholesterol removal from the broth media containing 100µg/ml of polyethanyl cholesterol sebacate ranged from 55-85%. The highest removal was shown by *Lactobacillus plantarum* YD5S as 85%, followed by *Enterococcus faecium* YHC12 as 80% (Fig. 1). The lowest percentage of cholesterol removal was shown by YD11S *Lactobacillus paraplantarum* to be 55%. Cholesterol as small as 1 m mol higher than the normal cholesterol level has been shown to increase the risk of coronary heart disease and coronary death by approximately 35 and 45%,
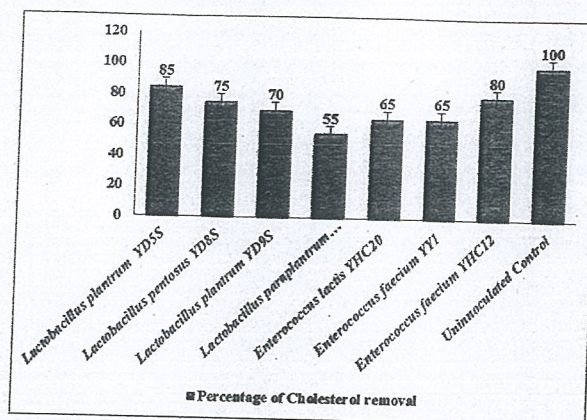


**Fig. 1:** Percentage of cholesterol removal by LAB strains

respectively (14). Reduction of total serum cholesterol of 1% can lower the risk of coronary heart disease by 2 to 3% (11). Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease (11). Although therapeutic drugs are available to relieve this problem, they are often expensive and can have side effects. Several studies indicated that *Lactobacillus* species were able to reduce cholesterol via several mechanisms (14, 19, 8, 26).

**BSH assay and deconjugation of sodium glycocholate and sodium taurocholate:** Only the BSH positive isolates were studied for BSH and other related assays. The BSH activity of LAB isolates from the fermented yak milk products have been shown in Table 2. All isolates have shown varying degrees of BSH activities towards both Sodium Glycocholate (SG) and Sodium Taurocholate (ST). Most of the isolates showed substrate preference towards SG compared to ST. The BSH activity in SG ranged from 0.82 U/ml to 1.30 U/ml. The highest activity was shown by *Enterococcus faecium*

**Table 2:** BSH activity on sodium glycocholate and sodium taurocholate by LAB strains

| | Sodium glychocholate | | | Sodium taurocholate | | |
|---|---|---|---|---|---|---|
| | Total protein mg/ml | Total activity U/ml | Specific activity U/mg | Total protein mg/ml | Total activity U/ml | Specific activity U/mg |
| *Lactobacillus plantarum* YD9S | 1.20±0.65 | 0.87±0.23 | 0.73±0.34 | 1.17±0.35 | 1.06±0.39 | 0.91±0.16 |
| *Lactobacillus paraplantarum* YD11S | 1.24±0.23 | 1.03±0.15 | 0.83±0.12 | 1.21±0.11 | 0.88±0.23 | 0.73±0.34 |
| *Lactobacillus pentosus* YD8S | 1.19±0.21 | 0.82±0.54 | 0.69±0.25 | 1.20±0.16 | 0.74±0.11 | 0.62±0.23 |
| *Lactobacillus plantarum* YD5S | 1.20±0.23 | 0.82±0.13 | 0.68±0.21 | 1.20±0.11 | 0.80±0.17 | 0.78±0.24 |
| *Enterococcus faecium* YY1 | 1.21±0.11 | 1.30±0.10 | 1.07±0.11 | 1.22±0.18 | 0.93±0.11 | 0.76±0.23 |
| *Enterococcus lactis* YHC20 | 1.21±0.56 | 0.92±0.32 | 0.76±0.25 | 1.24±0.19 | 0.83±0.19 | 0.67±0.15 |

Values are means of triplicates from two separate runs, n = 2. BSH activity from cell free extracts of LAB isolates grown on MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate

YY1 and minimum activity was shown by *Lactobacillus plantarum* YD5S and *Lactobacillus pentosus* YD8S. In case of ST, the BSH activity ranged from 0.74U/ml to 1.06U/ml. The highest activity was shown by *Lactobacillus plantarum* YD9S and minimum activity was shown by *Lactobacillus pentosus* YD8S. The highest BSH activity was shown by *Enterococcus faecium* YY1 in glycine conjugated bile. Specific activity of BSH did not correlate well with total BSH activity by most strains due to varying protein content in cell extracts. Highest specific activity was also shown by *Enterococcus faecium* YY1 in glycine conjugated bile while lowest activity was seen in *Lactobacillus pentosus* YD8S in taurine conjugated bile containing broth. In this study, all lactobacilli strains studied could deconjugate both glycine- and taurine-conjugated bile salts into cholic acid. However more glycine conjugated bile salt was found to be deconjugated by the strains belonging to *Lactobacillus pentosus* YD8S, *Lactobacillus plantarum* YD9S, *Lactobacillus paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *Enterococcus faecium* YY1. But incase of *Lactobacillus plantarum* YD5S the deconjugation of taurine conjugated bile was more than the glycine conjugated bile. Bile salt deconjugation ability by LAB isolates from fermented yak milk products is shown in Table 3. The bile acid deconjugation was determined by the amount of cholic acid released which ranged from 1.4 to 3.30 mM in SG and 0.49 to 1.98 mM in ST. All isolates were able to deconjugate bile salts, Sodium glycocholate and Sodium taurocholate at different degrees. YD11S *Lactobacillus* showed better deconjugation ability as compared to other isolates. In broth containing Sodium glycocholate highest deconjugation was shown by YD11S *Lactobacillus*

*paraplantarum* followed by YD5S *Lactobacillus plantarum*, YD9S *Lactobacillus plantarum*, YY1 *Enterococcus faecium*, YHC20 and YD8S *Lactobacillus pentosus*. Since sodium glycocholate predominates the human intestine, Brashears et al. (1998) (26) postulated that strains that prefer to deconjugate sodium glycocholate may have more potential to lower serum cholesterol concentrations if the deconjugation mechanism is important in decreasing serum cholesterol.

***Co-precipitation of cholesterol with deconjugated bile:*** Co-precipation of cholesterol with cholic acid as liberated from the deconjugation of Sodium glycocholate and Sodium Taurocholate by LAB isolates from fermented Yak milk product is shown in Table 4. Cholesterol was co-precipitated with deconjugation of both Sodium glycocholate and Sodium Taurocholate at varying degrees. Precipitation of cholesterol upon deconjugation of Sodium glycocholate ranged from 1.9 to 4 ìg/ml which was higher compared to Sodium Taurocholate which ranged from 1.4 to 2.3 µg/ml. Highest cholesterol precipitation in SG containing broth was shown by *Lactobacillus plantarum* YD9S, *Enterococcus faecium* YY1 followed by *Lactobacillus pentosus* YD8S as 3.2 ìg/ml. The lowest co-precipitation with deconjugation was shown by *Lactobacillus paraplantarum* YD11S as 1.9 ìg/ml. Cholesterol precipitation with Sodium Taurocholate by *Enterococcus faecium* YY1 was higher compared to other isolates. Highest cholesterol precipitation was obtained from *Enterococcus faecium* YY1 followed by *Lactobacillus plantarum* YD5S, *Lactobacillus pentosus* YD8S, *Enterococcus lactis* YHC20 and *Lactobacillus paraplantarum* YD11S with *Lactobacillus plantarum*

**Table 3:** Deconjugation of sodium glycocholate and sodium taurocholate by LAB strains

| Strain | Cholic acid released (mM) | |
| --- | --- | --- |
| | Sodium glycocholate | Sodium taurocholate |
| *Lactobacillus plantarum* YD9S | 1.97±0.24 | 1.96±0.21 |
| *Lactobacillus paraplantarum* YD11S | 3.30±0.35 | 1.98±0.50 |
| *Lactobacillus pentosus* YD8S | 1.40±0.26 | 1.25±0.28 |
| *Lactobacillus plantarum* YD5S | 2.47±0.11 | 0.49±0.10 |
| *Enterococcus faecium* YY1 | 1.79±0.10 | 0.66±0.26 |
| *Enterococcus lactis* YHC20 | 1.67±0.11 | 1.64±0.12 |

**Table 4:** Cholesterol precipitation with deconjugation of sodium glycocholate and sodium taurocholate by LAB strains

| Strain | Cholesterol precipitated (µg/ml) | |
| --- | --- | --- |
| | Sodium glycocholate | Sodium taurocholate |
| *Lactobacillus plantarum* YD9S | 4.0±0.34 | 1.4±0.12 |
| *Lactobacillus paraplantarum* YD11S | 1.9±0.23 | 1.7±0.34 |
| *Lactobacillus pentosus* YD8S | 3.2±0.15 | 2.1±0.21 |
| *Lactobacillus plantarum* YD5S | 2.9±0.15 | 2.0±0.17 |
| *Enterococcus faecium* YY1 | 4.0±0.11 | 2.3±0.16 |
| *Enterococcus lactis* YHC20 | 2.3±0.12 | 1.8±0.23 |

YD9S showing the lowest cholesterol precipitation.

**Cholesterol incorporation:** The strain that was grouped as BSH negative was tested for cholesterol incorporation into the cells. The percentage of cholesterol incorporation in *Enterococcus faecium* YHC12 was 72%. The cells of *Enterococcus faecium* YHC12 that was grown in the presence of oxgall and cholesterol micelles showed greater resistance to lysis by sonication than those grown in control broth. These results suggest that cholesterol may have altered the cellular membrane or wall of the lactobacilli so that they were more resistant to sonic disruption.

**Low pH/acid tolerance:** The pH of gastric juice secreted in the stomach is about 2 and many microorganisms are destroyed at this and lower pH. So, the tolerance to acidic conditions is a very important criterion for selection of probiotic bacteria (27). The pH 2.0 was regarded as a strong discriminative pH for the selection of high acid tolerant strains (28). The viable cell counts (log CFU/ml) and survival percentages of selected LAB to acid conditions at pH 2 after 2h incubation are presented in Table 5. The strain *Lactobacillus plantarum* YD5S and by *Lactobacillus plantarum* YD9S have shown their maximum ability to tolerate the acidic pH for 2h. For *Lactobacillus plantarum* YD5S the counts decreased by 5.5 log cycles and for *Lactobacillus plantarum* YD9S, the counts decreased by 5.83 log cycles, thereby showing greater acid tolerance over entire incubation period of 2 h. However in the rest of the isolates of *Lactobacillus* (YD8S, and YD11S) showed greater acid tolerance and their counts decreased by 2.17-3.29 log cycles as compared to 6.47-7.15 log cycles for *Enterococcus* (YY1, YHC20). For acid sensitive isolates, the viability decreased slowly for the first hour of incubation followed by rapid decline at the end of 2 h incubation period.

**Table 5:** Low pH or Acid tolerance LAB strains at pH2

| Strain | Viability count at pH 2(log CFU/ml) | | |
|---|---|---|---|
| | 0hr | 1hr | 2hr |
| *Lactobacillus plantarum* YD9S | 9.028±0.002 | 5.54±0.05 | 3.2±0.01 |
| *Lactobacillus paraplantarum* YD11S | 11.17±0.05 | 9±0.05 | 0 |
| *Lactobacillus pentosus* YD8S | 11.29 ±0.001 | 8±0.01 | 0 |
| *Lactobacillus plantarum* YD5S | 9±0.005 | 6.56±0.007 | 3.5±0.007 |
| *Enterococcus faecium* YY1 | 9.67±0.007 | 3.2±0.01 | 0 |
| *Enterococcus lactis* YHC20 | 9.36±0.004 | 2.45±0.007 | 0 |
| *Enterococcus faecium* YHC12 | 9±0.07 | 0 | 0 |

**Cell surface hydrophobocity:** The results revealed greatest hydrophobocity for n-hexadecane was observed for *Lactobacillus pentosus* YD8S, *Lactobacillus paraplantarum* YD11S, *Enterococcus lactis* YHC20, *Lactobacillus plantarum* YD9S and *Lactobacillus plantarum* YD5S, at 94.80%, 75.01%, 85.18%, 66.94% and 71.45% respectively (Fig. 2). These isolates may be considered as a potential probiotic culture from the adherence test.
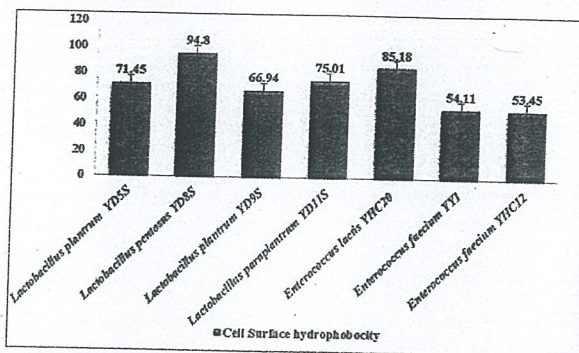


**Fig. 2:** Cell surface hydrophobocity of the LAB strains

The results of this study reveal that the possible mechanism(s) involved in cholesterol removal varied among the different strains of lactic acid bacteria. In contrast to previous studies, where BSH was considered to be the main mechanism for cholesterol removal, the strains tested in our study were able to reduce the cholesterol level even in the absence of BSH. Due to the cholesterol lowering activities of these strains they may be considered as of probiotic use in food and medical applications. This strain is being further assessed for possible *in vivo* safety and use as a culture for the development of new food products.

## Acknowledgement

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# Indigenous rearing practices of Yak and its multipurpose uses in the Sikkim Himalayas

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**Abstract:** Sikkim is a small part in Indian Himalayas that infuses great diversity with a rich ecological hotspot zone. Sikkim has four districts, the climatic condition of which ranges from subtropical in the South to tundra in the North. Amongst the mammals of Sikkim are the rare Himalayan Black Bear, Blue Sheep, Red panda, Snow Leopard, Musk Deer and Yaks. The Yak is an important multipurpose mammal of the alpine Northern and Eastern Sikkim region. The traditional knowledge of Bhutia community living in the high altitude has been passed on from generation to generation but has no written document. Qualitative data was obtained through self designed questionnaire, and face to face interviews and discussions in 35 households between June 2013 and October 2013 in East and North Sikkim. Study revealed the maintenance of Yaks, grazing pattern indigenous plants, health management practices, traditional breeding practices and processing technology of meat, milk and fiber. The milk and meat was processed into fermented products. Thara, Tema, Shuza/Shapjha or Phuicha (beverage) and value added products made from skin, hair and tail were documented for the first time from Sikkim. Besides yak acting as an ingredient for poverty alleviation and food insurance for the highlanders, it also projects an area of research on conservation and fermentation technology of traditional food items.

**Keywords:** Dzomo, Ethnic community, Fermented food, Multipurpose, Sikkim, Yak.

## I. Introduction

The eastern part of the Indian Himalayas highlights great diversity. This part of India has chilling cold climate as well as hot humid atmosphere with affluent flora and fauna (1). Sikkim is a part of the Eastern Himalayas with the differentiation in the ecosystems it is also called a biodiversity hot spot region (1). Sikkim is one of the least populated states in India, it lies 27° 05' - 28° 09' north and 87° 59' - 88° 56' east covering an area of 7.096 km². Sikkim is bounded by a stretch of three international borders viz. Tibetan plateau in the North, Nepal in the west, and Bhutan in the east and one national border, the Darjeeling district of West Bengal in the South. The temperate, alpine eastern Himalayan regions are home to about 90% of the yak population of Sikkim. The world population of domestic yaks is about 14.2 million, of which 71 000 yaks are present in India (2). According to the summary report on 18th livestock census 2007 of Sikkim state there are 6468 yak in Sikkim alone (3). The mountainous terrain hardly leaves any land for agriculture although these regions are rich in medicinal plants. There are four districts in the small state of Sikkim: North, South, West and East populated by a number of indigenous communities viz., Bhujels, Bhutias, Chettris, Dukpas, Gurungs, Kagateys (Yolmos and Sherpas), Kamis, Lepchas, Limbus, Lachenpas, Mangars, Mistris (Sarkis), Newars, Rais, Tamangs and Thamis residing in this landscape for at least the past several centuries (1).

The Bhutias and Tibetan Dukpas are the major communities involved in yak herding after the twentieth century with herding being transferred from Eastern Nepal to Khangchendzonga landscape (5). Yak rearing is the only alternative to provide sustainable source of income to the people of the alpine region. The local communities herd livestock like yak, *Dzomo* (cow-yak hybrid) and domestic cattle. In Sikkim, yak is found at Lachen, Gurudongmar Lake, Chopta valley, Lachung valley and Yumthang in North district, Tsomgu Lake, Kupup, Thegu, Nathang in East district, and Yuksom-Dzongri in West Sikkim region. The first part of the paper describes the rearing of Yak by the ethnic communities in North and East Sikkim. However, the rearing of Yak originally started from Nepal, which was inherited from Bhutan (5). The Second part consists of its multipurpose uses; being raised by the herders for providing milk, meat and manure. Using the traditional knowledge passed on from generations the herdsman has developed novel practices for yak husbandry and yak product technology.

The present study describes the interrelationship that exists between yak and human that include the small group if ethnic people of North and East Sikkim. The knowledge gap that exists between local people and future generations can be filled by the preservation of their true culture, social and ecological attributes. The paper would open new avenues for future research serving as a baseline for further research in Sikkim. Some new ethnic beverages, food and value added products have been studied and reported. It would also draw attention to the higher authority on keeping yak in high priority conservation.

## II.     Materials and Methods

Study was based on the most common research method. It is probably the best method for collecting original information of large groups. Through the method of open question and face to face interviews it was easier for the interviewer to explore for additional information on qualitative data collection from the respondents to ensure the facts on traditional yak management practices and the processing technology of the traditional products from yak milk. A set of questionnaire was prepared for the study area. The Tsmogu lake region in the East District, Lachen and Lachung in North District of Sikkim were the study areas, as maximum yak populations are found in these regions. A random sample of 35 households were considered, those involved in yak rearing and using traditional practices in maintaining their livelihood as well as the neighboring households. The management practices adopted by the herder community, the production of milk products and milk processing methods were studied in these areas. During the inquisitiveness of seeking facts from the herdsman, there was explanation on the purpose of research, benefits, research methodology used for the aim to preserve and conserve the traditional knowledge.

## III.     Results and Discussions

The word "Yak" is an English word derived from Bhutia language *gyag*. Yak word in English refers to both male and female but in Tibetan, male is called yak and female is called *De*. The hybrids of yak and cattle are called *dzomo* (female) and *dzo* (male). The ancestors of yak were from Tibet which then migrated to Bhutan and Nepal and have however developed characteristics due to evolution (4). The scientific name of yak is *Bos gruinnens* belonging to Class Mamalia and Phylum Chordata, with a height of about 2 meters and long hair covered body to keep them insulated in the cold alpine regions of the Himalayas. The various characteristics of yak must be attributed as adaptations to many factors like extreme cold; high altitude with low oxygen content of the air and nutrition with short growing seasons for grazing in herbaceous plants (5). Yak survives in the annual mean temperature below 5°C and the average in the hottest month is less than 13°C (5). This bovine mammal can survive cold temperatures up to – 40 °C and altitude of 3000 – 5000 m above the mean sea level. As per traditional knowledge, yak survival in such extreme condition is only due to the presence of long thick hair on their bodies, and rich fat layer deposit which keep them to remain insulated in such low temperatures. In the winter season the yaks maintain their internal body temperature by the presence of long coarse outer hair and an undercoat of fine hair. It is believed that yak adapt to low oxygen content of the air as they possess a large chest, large lungs and a large heart relative to their overall body size. In the Northern region it was observed that yaks differ in the amount of hair in their body, the color of body hair, milk production and presence or absence of horns (Figure 1).

According to the herders, wild yak prefer to live in herds of tens or even hundreds of animals, they have a very acute sense of smell and try to escape immediately on sensing or seeing people or other animals. According to the herders wild yak is large in body and strong, they have thick and long hair covering the whole body. The color of the hair is usually jet brown or jet black. Wild yaks are highly tolerant of cold and starvation. Wild Yak is resistant to diseases as reported by herder community but diseases due to parasitic infections through ticks and lice are common.

Yak is also called "surefooted animal" and excellent pack animal (8) as its foot has major grip towards the bumpy and stony heights of the rocky mountain terrain and for this reason it is often used to carry ration and other goods from one part to another by the tribes and army men to their camps in the North Sikkim which cannot be reached by horse or sheep. The yak bull when crossed with local cow produces *Zo* which is mainly used to carry goods. Yak, if in danger of sinking in a marsh, will spread out their legs and use the inner side of their bodies to escape from sinking; it will show swimming-kind of motion rather than panic and thrash around as a horse might (8). The strong limbs and solid hooves of yak attributes help them to walk in dangerous places and over marshland and to climb over steep mountains.
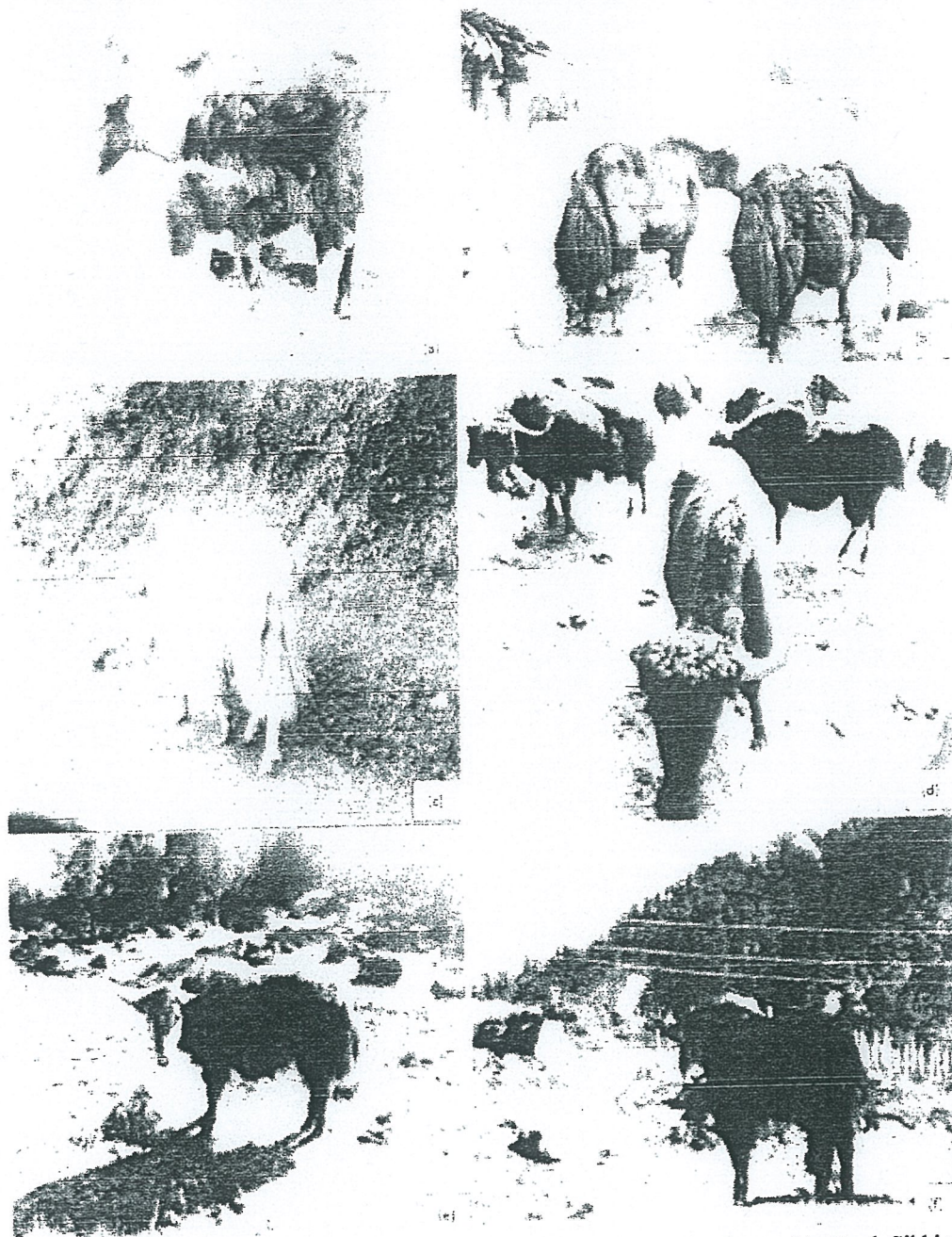
**Figure 1** (a), (b), (c) and (d) Yaks observed in East Sikkim, (e), (f) Yaks observed in North Sikkim

## 1. Yak rearing

### 1.1. Management and grazing pattern of Yak

The herders generally follow traditional methods of yak management owing to the topography and cultural influences. The yak raisers or the herdsman maintain yak in a semi-migratory fashion. During the winter seasons the yaks survive in extreme cold conditions but due to decreased vegetation they are moved down to lower areas with better pasture. When summer arrives and the temperature starts to warm up the yaks are migrated to higher altitudes into the temporary houses. The local herders train yak to understand commands by throwing stones in sling or *whurto*, made from its coarse fiber to control yak herds and chase unwanted animals. Yak grazes in open areas that are not fenced, after grazing yaks are called by herders by whistling or singing. At night yaks are brought to the shed for protection from wild animals and for milking. In summer season yaks are

left free for grazing early and brought late to the shed but during the winter season yaks go out late and brought back early. The herders have indigenous traditional ecological knowledge for selection of diverse groups of native shrubs. Being a herbivore animal, yak feeds on wild flowers and various shrubs in the alpine Northern Sikkim State. Yak depends 90-95% on the grazing pattern. It was observed that yaks graze on long grasses like other cattle do but they can also graze on short grass, creeping stems, roots and tender branches or shrubs. The yak feeds on palatable shrubs like *Kobresia napalensis* ("sun buki"), *Kobresia capillifolia* ("kesari buki"), *Carex nivalis* ("dharkhare"), *Phleum alpinum* ("doodhe jharr"), *Festuca valesiaca* ("rani buki"), *Kobresia duthiei* ("bhalu buki"), *Juncus* sp. ("suire buki"), *Allium prattii* ("dandu"), *Heracleum* sp. ("ganer"), *Selinum tenuifolium* ("cheeru"), *Rheum acuminatum* ("khokim") and *Carex nigra* ("harkat"), during the summer season. The indigenous herbs besides having medicinal attributes are considered by the herders to be nutrient rich (5). It is believed that in some households the herds are kept together and moved from less grazing pastures to more grazing pastures. But in some households yaks are kept in fenced areas where they are grazed in a surrounding area and taken to the shed for feeding. Apart from grazing yaks are fed with supplementary feed like molasses, barley, maize flour and water. The lactating herd, non- lactating herd and the pack herd are kept separately in the shed. Yaks consume less feed than cattle, preferring fresh and high quality forages; their feed intake gets reduced at high temperature. Yaks are also fed with bamboo leaves cut from the local areas by the owners. The sheds are cleaned every morning after sending the yaks for grazing in pastures, the feces are collected in a bucket and the uneaten feed is removed. The feces are pressed with fingers and then dried for future use as fuel for cooking.

### 1.2. Indigenous practices for yak health management

Many diseases in yak are thought to be caused by nutrient deficiency especially during winter and also by environmental stress. Yak and cattle are affected by similar kinds of diseases. The herders informed that yak feed on the dead remains of animals that harbor harmful bacteria often causing infection. Yak is also sometimes infected by polluted water. Blood and stool samples are collected by the officials of Department of Animal Husbandry and bacterial infections that result in symptoms of mineral drenching and food poisoning. Traditionally, affected animals are given a drink made from medicinal herbs. The Department of Animal Husbandry also supplies necessary antibiotics and vitamins for these kinds of infections. The herdsman said, under certain circumstances of infection symptoms of abortion also appear. Mastitis occurs in yak, but it is believed that the incidence is less than among dairy cattle, because of the relatively low milk yield of yak and the suckling of calves. Pal (1993) referred to a virus outbreak in Sikkim in 1973(6). It is also believed that yaks are affected by diseases caused by parasites like ticks, lice and mites. Sometimes yaks die after drinking marshy stagnant water especially during the onset of the winter season. Yaks are also affected by diarrhoea; the stools are of a thin, pasty to watery consistency and sometimes contain blood. The herders fed the yaks with drink made from traditional herbs to alleviate such symptoms.

Some herdsman feed them with wheat and salt adding to mineral content when they do not have balanced mineral supplement after every 15 days. It is said that, the milk quality depends on the medicinal property of the herbs and shrubs of the terrain. The herder narrated that milk yield is closely related to pasture growth and quality. The yaks are fed with the residue of Chhurpi and Shyow as they are highly nutritious. After giving birth to a young calf, yak is fed with residue of local alcoholic beverage made from rice, maize, barley and finger millets. They are also fed with jaggery for quick recovery from child birth. The herdsmen use crush rhizome of a local medicinal herb locally called as "pakhanved" (*Bergenia ciliata*) to cure fractured bone, fresh cuts and wounds of yaks.

### 1.3. Breeding of Yak

As said by the herder community the breeding season reaches at peak during July to August when temperature is at its highest and grass growth at its best thereafter stops around November. Yak breeds in the warmer months with a gestation period of nine months in general like cow and give birth to young calf during April to July with May being the peak month, however the gestation period may vary with respect to the type of yak. Most yaks give birth to young calf at 5 to 6 years of age. In three years there are 2 calves as informed by the herders. The body weight of young born calf is estimated by herders to be about 10 kg to 16 kg whereas the adult body weight is 150 kg to 175 kg. It is believed if the male has a thick and a short horn then the chances of producing male is high. The life span of wild yak is believed to be about 20 years. During the survey, the herder informed that in earlier days when Sikkim was an independent country, there used to be frequent trade between Tibet and Sikkim as the border was porous. The open movement across the border facilitated cross breeding of yaks. But when the borders were sealed, the herders faced problems of inbreeding which resulted in weak, unhealthy and unproductive stocks. As informed by a personnel from the Animal Husbandry Department, Government of Sikkim, crossbreeding practices was done to improve the quality of yak but it was not successful when elite yak bull from Bhutan was supplied to the farmers for improving the genetic traits and conservation of this threatened breed in the period of four years. However Yak breeding farms have been developed at Chopta

valley as a summer station and another breeding centre at Zeema in North Sikkim as winter station by the state Government.

### 1.4. Milk Processing

It is believed that milk yield is higher in hybrids than in pure yak. *Pheno* refers to yak type in Bhutia language gives less milk compared to *Hazi* type which gives more milk. The consistency of yak milk is thick, fatty, cream in color and sweet in taste. Total yak milk yield is 147-487 kg during lactation period of 180 days has been reported (9). Yak milk and dairy products are popular foods in high-altitude regions. Yak milk contains 16.9- 17.7% solids, 4.9-5.3% protein, 5.5-7.2% fat, 4.5-5.0% lactose, and 0.8- 0.9% minerals (9). Yak milk is rich in fat, protein, essential minerals, and healthy polyunsaturated fatty acids such as conjugated linoleic acid and omega-3 fatty acids (10, 11). Yak milk and its milk products play a major role in providing essential nutrients and minerals to the herdsman and the native people of the region as a staple food. The milching is done by the women once during the early morning and in some cases twice also in the evening before the sun set when the cattle return from the pasture. For milking the hind legs of yak are tied and then milking is started in the shed. Sometimes women also go to the yak around the pasture with bucket in hand for milking. The amount of milk driven by hand ranges from ½ litre to 2½ litre with the peak milk yield during the months of August and September. Collected milk is then preserved before being molded into various value added products to earn their livelihood by the implementation of their indigenous knowledge. Milk is collected and kept without boiling in utensils made up of wood and sometimes plastic jars for several days. Only when the sufficient milk is collected then processing of milk is done. Although milk is taken at the expense of calf, yak milk is molded into varieties of indigenous milk products in various household.

## 2. Multipurpose uses

### 2.1. Milk products

The most common Yak milk products of Sikkim are *Shyow* (Dahi in Nepali) (7), *Marr* (Ghew in Nepali) (7), *Thara* (Mohi in Nepali), *Chhurpi* (7), Chilu (Yak Fat), *Philu* (7) and *Tema* (Yak cream) (as per survey) (Table 1).

Table 1.The various traditional products of yak milk.

| Sl. no | Local names of yak milk products | Common names in English |
|--------|----------------------------------|-------------------------|
| i) | *Shyow* | Curd |
| ii) | *Khachu* | Whey |
| iii) | *Marr* | Butter |
| iv) | *Shuza/Shapjha or Phuicha* | Butter Tea |
| v) | *Chhurpi* | Cheese |
| vi) | *Philu* | Creamy cheese |
| vii) | *Tema* | Cream |

#### 2.1.1. *Shyow*

*Shyow* is curd in Bhutia language, it is known as Dahi in Nepali or Hindi language. *Shyow* is either naturally fermented yak milk or is prepared from the addition of a starter culture used for fermentation (7).

#### 2.1.2. *Khachu*

Butter milk or whey is known as *Khachu* in the local Bhutia language and *Mohi* in Nepali. It is the fermented by-product of *Shyow* or Dahi. It is a popular refreshing beverage of the alpine region.

#### 2.1.3. *Marr*

Butter is known as *Marr* in the local Bhutia language and it is known as *Ghew* in Nepali (7). It is one of the principal yak milk products of the local community of North and Eastern alpine region of Sikkim and highly expensive being Rs 700- Rs 800/- per kg. The herder communities churn *Shyow* in wooden containers locally called as *zodong*. Some of the solid butter is kept for consumption and some for offering to the sacred Gumpa (Temple) in the form of butter lamps (Figure 2a). *Marr* is used for cooking and frying edible items, or it is consumed directly. It is used to prepare *Tsamba*, made from barley is the staple food of the Dokpas that looks like flour. *Marr* is melted and added to barley and baked potato by the local people and consumed as a snack.

#### 2.1.4. *Shuza/Shapjha or Phuicha*

The local people consume tea made up of yak milk with salt known as *Shuza/Shapjha or Phuicha* in the local language of the herders. It is believed to be one of the oldest traditional beverages of the indigenous community in the alpine region. They drink tea throughout the day providing the Dokpas the most possessed supplement in their diet. Salt tea has been most popular delicacy among the local people and consumed on a

regular basis although sugar is available to them. In Nepali language it is called *Ghew Chea*. It is a refreshing beverage served by the local community to their guests.
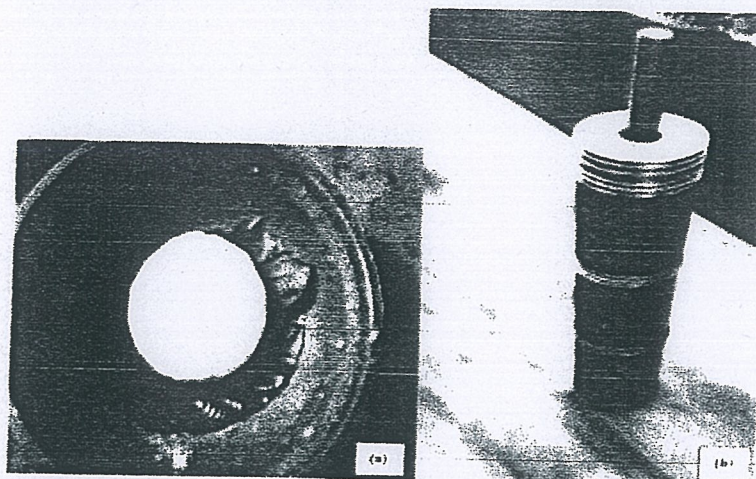


Figure 2 (a) *Marr* or Butter ready to be packed into leaf (b) *Dongmo,* vessel for making *Shuza/Shaviha or Phuicha*

It is made in wooden vessel known as *Dongmo* by herders (Figure 2b). Boiling tea is added in Dongmo, *Marr* (Butter), salt and yak milk is added for taste and spun with a wooden stick tied to rope by hand. The tea has a soup like consistency and is served hot.

### 2.1.5.  *Chhurpi*

*Chhurpi* is of three types:  Soft *Chhurpi* , Hard *Chhurpi* and Dudh *Chhurpi* (7). Soft *Chhurpi* is one of the most common traditional fermented food products in the house hold of the Sikkimese community. To the local this delicacy item is highly palatable and also replaceable to even the non-vegetarian food. Hard *Chhurpi* is sweet in taste and chewy. It is the most famous ethnic food highly relished by the tribal community. These types of *Chhurpi* are very hard and have low moisture content; it can be stored for a number of years. The more aged the harder it is with more value. *Chhurpi* costs about Rs 600/- per kg in the surrounding area and even more if marketed (Figure 3a). *Dudh Chhurpi* (Figure 3b) is much expensive than other two *Chhurpi*.
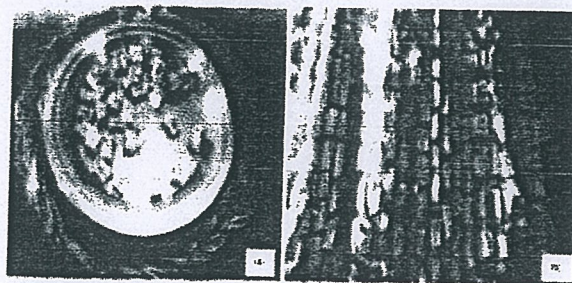


Figure 3 (a) Hard *Chhurpi* (b) *Dudh Chhurpi*

### 2.1.6.  *Philu*

During milking of yak, fresh the milk is added directly into cylindrical bamboo vessels (called *dzydung* by the Bhutia) or in wooden vessels (called *yadung*). The soft creamy mass attached to the branches of *lawa*, a local plant is then scraped off and stored as local cheese which has great value in the market. This soft creamy mass is called *philu* (7).

### 3.1  Meat Products

Meat forms a major part if the diet of the local people in the North and Eastern part of Sikkim. The local people prepare and consume a variety of traditionally processed smoked, sun-dried, air-dried, or fermented meat products, including sausages since olden days. Male yak is slaughtered instead of female yak. Mostly yak killed in accidents is consumed by the herdsman. The herdsmen consume yak meat on a daily basis and also

during festivals like *Losar* in month of February (Figure 4). The following are the yak meat products: *Satchu* (Dry meat), *Kargyong* or *Gyuma* (Sausages) and *Chilu* (Yak Fat) (11). Yak *Satchu* costs Rs 500 to 600 per kg. According to one of the herdsman, in olden days fat separated from fresh meat was collected in sheep stomach, pressed with stones and hung in their houses but nowadays they use wooden jars to store fat until further use. Food cooked in its fat is considered as being tastier than in normal oil. Fat stored in these manner are used by the local community for a year or more.
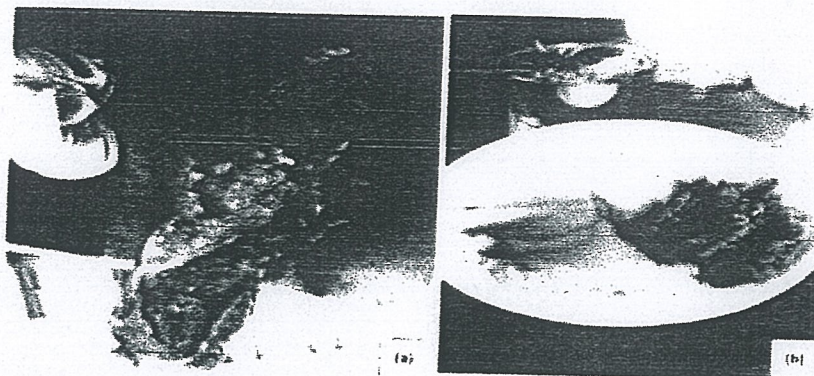


Figure 4(a) Yak meat in normal drying process, (b) Cooked meat served with "T momos"

### 4.1. Value added products from Yak Fibre

Two types of wool are found in yak, coarse outer hair and the inner soft hair coat called *Khullu* in Bhutia language. During shearing Yak gives more coarse hair of about 2 kg and 1/2 kg of fine hair. The hair of Yak is highly water proof with great tensile strength (Figure 5 (a, b).
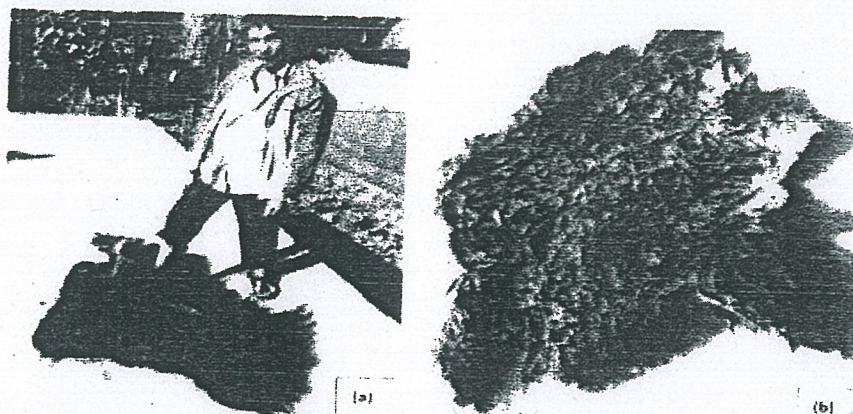


Figure 5 (a and b) Yak fibre, drying of detached the skin with hair from the yak body

The horns of Yak are also used for decorative purposes and considered holy. The horns are round and very thick about 15 - 20 cm in diameter. Fine wool or *Khullu* is used to make garments like muffler, sweaters and blankets. The fine wool woven to make garments resists rain and cold winter. Coarse hair is used to make tents, caps, blankets, hand bags, door mats and hand woven carpets. Carpets (Figure 6a) and door mats are woven in wooden frames traditionally and are used to cover the floor, chairs for making softer and warmer sitting in chairs. Good quality caps are also made by mixing the yak wool with the fine wool of Angora rabbit making them highly attractive, aesthetic and acceptable by the tourists. Ropes are prepared from long hairs that are twisted over to tie tents or domestic animals. The ropes are highly strong that can withstand sun, wind and rain. Slings locally known as *whurto,* made from coarse fibre is used to control yak herds and chase unwanted animals. Besides body hair yak tail also has religious value. The tail is washed properly and tied tightly with a rope in a wooded handle to make *Chamar* and used as a fly whisker in some areas in India. Besides a fly whisker it also has some aesthetic value. Its cost price in local markets ranges from Rs 5,000 to Rs 6,000 (Figure 6b). Yak skin is also used to make hide, tents to resist cold, *mura* or stool (Figure 6c) and for many decorative purposes.
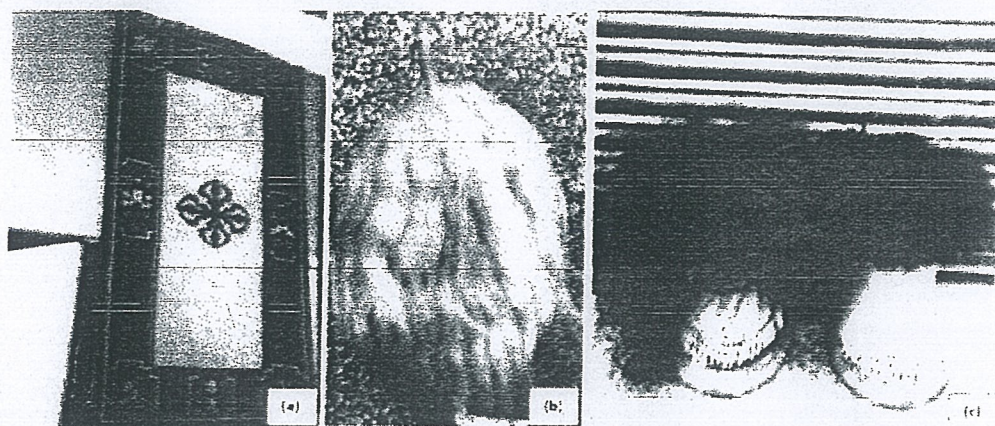
Figure 6 (a) Carpet made from Yak hair, (b) Yak tail or *Chamar* and (c) Yak hair attached with skin to make *mura* or stool.

## IV. Conclusion

The herder community follow traditional pattern of management and grazing pattern of yaks due to climatic conditions and socio- cultural conditions. The yak keeping varies from both primitive and advanced ways of semi- migration in winter seasons. Grazing pattern followed by the herders depend on their indigenous knowledge of pasture. The traditional health management practices rely on the knowledge of indigenous medicinal herbs passed from generation to generation. The indigenous knowledge all together plays a pivotal role in maintaining yak sustainability and also their livelihood sustainability. Thus the documentation of traditional knowledge would provide the preservation of culture and indigenous practices used by the Lachenpas, Lachungpas, Dokpas and Bhutia communities of North and East Sikkim and used as a key for poverty mitigation and food security. The livelihood sustainability of the herdsman of the alpine Sikkim is mainly around the Yak, a "surefooted" multipurpose animal. Yak is their major income generating source for poor herdsman from its milk, meat, hair, skin to tail serving them to help their survival in this world away from transformation making them to value traditional values, religion, community and ethnic beliefs. Without it, one cannot imagine how humans could survive in this beautiful but hostile environment.

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# Questionnaire

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**BUDDHIMAN TAMANG**

1. Name of the Respondent


2. Places where Yak population in the Sikkim Himalayas?
   East Sikkim:
   North Sikkim:


3. What are the different types of Yak species in Sikkim?


4. Description the Yak with respect to the approximate size, height, weight:


5. How breeding is done?


6. Discussion of Yak as a livelihood to the herder community in the Sikkim Himalayas.


7. How is the feeding pattern of Yak in the higher altitude?


8. What are the indigenous trees and shrub species as fodder for the Yak?


9. Average amount of milk obtained from a yak. _____ (lowest_____; Highest_____)


10. Any differences in milk yield on species/breed?

11. How is the quality of milk and the milking process in Yak?

12. How yak milk is consumed? Is it for own use or sold?

13. What are the products made from yak milk? Describe the products.

14. Describe the methods of preserving the Yak meat products?

15. What are the different kinds of wool obtained from Yak?

16. How Yak serves as a multipurpose animal?

17. What is the demand of yak prducts in the market in Sikkim and outside Sikkim

18. What are the indigenous practices for Yak health?

19. What are the programmes taken by the Government in the preservation of these Yak species in Sikkim?