To Study the Antibiotic Susceptibility Pattern of Isolates of Lactic Acid Bacteria from Gut of Healthy Individuals

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Nilu Pradhan

Roll No: 12PDMB05

Registration No: 12SU6399

Department of Microbiology, School of Life Sciences

Sikkim University

Tadong-737102

India

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DEPARTMENT OF MICROBIOLOGY

Sikkim University

(A Central University established by an Act of Parliament of India, 2007)

6th Mile, Samdur, P.O Tadong 737102, Gangtok, Sikkim, India

***Buddhiman Tamang**, PhD*

Assistant Professor

CERTIFICATE

This is to certify that the thesis entitled **"To Study the Antibiotic Susceptibility Pattern of Isolates of Lactic Acid Bacteria from Gut of Healthy Individuals"** submitted to the **Sikkim University** for the award of **Master of Philosophy Degree in Microbiology,** embodies the results of *bona fide* research work carried out by **Ms. Nilu Pradhan** under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate-ship and fellowship.

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

Place: Gangtok, Sikkim

Date:.... 29/08/2014

Dr. B. M. Tamang

(Supervisor)

Phone: 03592-232085; E-mail: bmtamang3@gmail.com

Website: www.sikkimuniversity.ac.in

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DECLARATION

I hereby declare that the thesis entitled **"To Study the Antibiotic Susceptibility Pattern of Isolates of Lactic Acid Bacteria from Gut of Healthy Individuals"** submitted for the award of **Master of Philosophy (M.Phil.)** Degree in Microbiology of Sikkim University is a record of my original work done by me. The content of this thesis is based on the experiments which I have performed myself. This thesis has not been submitted for any other degree to this University or any other University.

Date: 29/08/2014

Nilu Pradhan
Nilu Pradhan
Roll No: 12PDMB05
Regn.No: 12SU6399

We recommend that the thesis be placed before the Examiners for evaluation.

(Dr. H. K. Tiwari)
Head, Department of Microbiology

Head
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
Gangtok-Sikkim

29/8/2014
(Dr. Buddhiman Tamang)
Supervisor

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757
6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com

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NILU PRADHAN

Dedicated to my beloved Parents and Sister

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LIST OF ABBREVIATIONS

1. CFU -Colony Forming Unit
2. CLSI- Clinical and Laboratory Standards Institute
3. LAB- Lactic Acid Bacteria
4. LSM- Lactic Acid Bacterium susceptibility test medium
5. MHA- Muller Hinton Agar
6. MIC- Minimum Inhibitory Concentration
7. MRSA- De Man Rogosa Sharpe Agar
8. ml- millilitre
9. mm- millimetre
10. MTCC- Microbial Type Culture Collection Centre
11. NA- Nutrient Agar media
12. pH- power of H ion concentration
13. SD- standard deviation
14. %- percentage
15. (w/v) - weight per volume
16. µg- microgram

Chapter - I

Introduction

1. Introduction

The Lactic Acid Bacteria (LAB) are Gram positive, catalase negative, anaerobic but microaerophilic, non-sporing (rods and cocci) and usually non-motile. LAB belongs to the phylum Firmicutes. The genera of LAB are *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus,Tetragenococcus, Vagococcus* and *Weissella* (Ercolini *et al.*, 2001; Jay, 2000; Holzapfel *et al.*, 2001; Stiles and Holzapfel, 1997). The functional Kreb's cycle is not present in LAB as it lack electron transport systems and cytochromes (Batt, 2000) and hence fermentative catabolism of carbohydrates (Kandler and Weiss, 1986) are the only means of generating energy in the LAB. On the basis of end product generated during carbohydrate metabolism, LAB are divided into two broad groups: homofermentative and heterofermentative (Jay, 2000). The common feature observed in LAB is the production of lactic acid as the end product from fermentation of carbohydrates (Ringo*et al.*, 2001).

LAB are distributed in a diverse ecology and habitats such as soil, water, sewage (Holzapfel *et al.*, 2001), food sources of both plant and animal origin and commonly on gastrointestinal tract and uriogenital tracts of animals and humans (Tannock, 1990). *Lactobacillus* sp. in the human faecal samples has been detected as high as 10^9cfu/g (Mitsuoka, 1992; Kimura *et al.*, 1997; Tannock *et al.*, 2000). The count of different genera and species of LAB varies in different sections of the gastrointestinal tract of normal healthy humans (Reuter, 2001; Sgouras *et al.*, 2004).

Wide variety of microbes residing in the gut has intense influence on human physiology and nutrition and are essential for human life (Hooper *et al.*, 2002); Ley *et al.*, 2008). In general, gut microbiota enhance the host immune responses (Neish, 2010). The LAB has been found to prevent various GI infections by forming a colonization barrier (van der Waaij *et al.*, 1973) against the establishment of microbes with pathogenic potential (Nader de Macias *et al.*, 1992; Zheng *et al.*, 1994). LAB are also associated with reduction of allergic reactions in pregnant women and in neonatal allergies (Rautava *et al.*, 2002). A wide variety of reactive oxygen and nitrogen species (ROS, RNS) are continuously produced in the human body, playing a substantial role in the pathogenesis of cancer, cardiovascular diseases, allergies, and atherosclerosis (Agerholm-Larsen *et al.*, 2000). LAB may have roles in prevention of

these diseases since antioxidative effect of *Lactobacilli* has also been reported (Kaizu *et al.*, 1993; Lin and Yen, 1999; Lin and Chyang, 2000).

There have been reports that food related LAB could act as reservoirs for antibiotic resistance genes (Franz, 2005; Klein, 1998). Therefore, the bacteria in food can act as a vehicle for antibiotic resistances, which might be transferred to commensal and pathogenic bacteria (Hummel *et al.*, 2007). LAB frequently possesses plasmids of diffferent sizes consequently; the antibiotic resistance determinants in plasmids have been reported in *Lactococcus lactis* and various *Lactobacillus* and *Enterococcus* species (Gevers *et al.*, 2003). Subsequently, LAB are found to be naturally resistant to many antibiotics (Lavanya *et al.*, 2011). The intrinsically resistant LAB (probiotic) strains to antibiotics might have beneficial effects on patients whose normal intestinal flora has become disturbed or unbalanced due to excessive use of antimicrobial agents (Sanders and Huis in't, 1999).

In general, LAB are highly susceptible to erythromycin, tetracycline (Ammor *et al.*, 2007) and ampicillin (Katla *et al.*, 2001) unless some pressure is created due to use of antimicrobial agents (Rao and Halami, 2012). Natural resistance to glycopeptides has been reported in *Lactobacilli*, *Pediococci* and *Leuconostoc* sp; the resistance mechanism is explained to the presence of D-ala-D-lac as the normal peptide in the peptidoglycan (Florez *et al.*, 2005). There are also reports that number of *Lactobacillus* sp also has natural resistance genes to kanamycin and streptomycin (Beta-lactams) (Curragh and Collin, 1992).

Currently, though there are sufficient data available about the prevalence of antibiotic resistance and the mechanisms involved in pathogenic bacteria, but inadequate information is provided about the antibiotic susceptibility or the existence of antibiotic resistance genes in commensal bacteria such as LAB isolated from human faeces (Teuber *et al.*, 1999; Catalouk and Gogebakan., 2004; Florez *et al.*, 2007; Zhou *et al.*, 2005) and less data do exist about antibiotic susceptibility.

The objective of this work is to assess the susceptibility of LAB isolates from the stool of healthy human to different antibiotics. Therefore, the sole aim of this study is to evaluate the current resistance or susceptibility patterns of LAB isolates. As per our knowledge study in this field has not been done in this part of country.

Chapter - II

Aims and Objectives

Chapter - III

Review of Literature

3. Review of Literature

The infant gut is sterile at the time of birth, immediately after birth range of microbes (facultative and strict anaerobes) begins to colonise the gut (Mitsuoka *et al.*, 1974). The gastrointestinal microflora of human consists of hundreds of diverse microbial species surviving ecologically diverse environment (Moore *et al.*, 1974). The presence of normal flora of human GIT are restricted to small and large intestine, *Bifidobacterium* although exceed in number than *Lactobacilli* (Finegold *et al.*, 1983).

The microflora itself is made up of hundreds of different species of microorganism (Moore and Holdeman, 1974). The estimation has been made that human colon contains $>10^{11}$ bacterial cells per gram contents approximately, 400 species (Conway, 1995; Savage, 1997). As such, the microbial population of GIT are much diverse, well adapted metabolically and can be called as renewable organ in body (Roderick, 1999). In the healthy host, colonisation of the alimentary canal by enteric microorganisms begin almost immediately after birth subsequently, the intestinal microflora composition remains comparatively constant (Eamonn and Rodrigo, 2006). The diversity of gut microflora is determined by various factors such as diet, genetic background and host physiological state (Eamonn and Rodrigo, 2006).

The GIT is an extended tube extending from lips to anus, divided into various defined anatomical regions (Roderick, 1999). In most of the papers on gut microbiology, small intestine, large intestine and faecal material are generally discussed, as it is easy to be obtained (Roderick, 1999). The microbial composition in stomach and proximal small intestine is relatively low due to peristalsis and effects of gastric juice, when present; the bacterial species are generally *Lactobacilli, Enterococci*, oral *Streptococci* and other obligate or facultative anaerobic gram positive bacteria which are supposed to be the original flora of oropharynx (Eamonn and Rodrigo, 2006). In normal human and animal, LAB are found in large numbers (Sgouras *et al.*, 2004).

The term 'autochthonous' and 'allochthonous' is used to represent the organisms present in the body. The organisms found at a particular site represent an indigenous or normal microbiota of that site; and are termed "autochthonous" (native to the place where they are found) species (Savage, 1977). The term "indigenous microbiota" also includes archaea, viruses, fungi, and protists present on the body's surfaces (Wilson, 2005). Autochthonous microbes occupy particular niches and are habitually found in

the human body (Savage, 1977). Allochthonous microbes in contrast are the transient flora and colonize particular habitat only under abnormal conditions, they enter the host from external environment (Savage, 1977). Most pathogenic microorganisms are allochthonous and reside harmoniously with the host, but it could be pathogenic, when the environment get disturbed (Trenschel *et al.*, 2000).

2.1 Lactic Acid Bacteria

LAB are gram-positive, non-sporulating and catalase negative rods or cocci that ferment various carbohydrates mainly to lactate and acetate. LAB are heterogenous group of bacteria, it comprises 20 genera within phylum Firmicutes. The genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are considered as the principal LAB (Holzapfel *et al.*, 2001). A feature common to all LAB is their ability to ferment hexose to produce lactic acid as the main end product. LAB are fermenting organisms, they lack cytochromes, electron transport system and Kreb's cycle (Batt, 2000).

LAB are divided into two groups, homofermentative such as *Lactococcus* and *Streptococcus* and heterofermentative such as *Leuconostoc* and *Weisella* sp, based on the end products of glucose metabolism (Jay, 2000). The group comprises cocci (*Streptococcus*, *Pediococcus* and *Leuconostoc*) and rods (*Lactobacillus* and *Bifidobacterium*), which are either entirely (homofermentative) or around 50 % (heterofermentative) and lactate producers (Kandler, 1983).

Various amino acids, vitamins and minerals are essential for their growth, LAB preferably grow in carbohydrate rich environment (Kandler *et al.*, 1986). Accordingly, they are commonly associated with nutritious environments like foods, decaying material and the mucosal surfaces of the gastrointestinal and urogenital tract (Kandler *et al.*, 1986; Havenaar *et al.*, 1992; Walstra *et al.*, 1999), where they enhance the host protection against pathogens (Havenaar *et al.*, 1992). They can also be found in plants or material of plant origin, fodder, fermented food (yogurt, cheese, olives, pickles, etc.) and also in the oral cavities (Hammes *et al.*, 1995). Broadly, LAB can be referred as a pioneering organism that colonises the digestive tract in great numbers (Tannock, 1990).

LAB were considered among the first living organisms to be present on the earth. They are assumed to have appeared on the transition period approximately three billion years ago from anerobiosis to aerobiosis. Since they bear all the required enzymes involved in respiration pathways, they seem to have adapted to both the aerobic and anaerobic conditions. However, during evolution they failed to acquire heme, component of cytochromes, so they require an external supplement of hemine to shift their respiratory metabolism. (Carr *et al.*, 2002). Therefore, LAB lacks an ability to get functional heme enzymes such as peroxidises and catalases, the proteins involved in hydroxyl scavenging, which results in impaired resistance to O_2 toxicity. Nevertheless, LAB can tolerate oxygen and are known as microareophilic organisms (Brioukhanov and Netrusov, 2007).

2.2 LAB in Human GIT

LAB are ecologically diverse group of microorganisms found in nature, which produce lactic acid as the primary product of sugar fermentation (Carr *et al.*, 2002)Among the other members of normal flora in gastrointestinal tract (GIT), LAB are a common microflora that inhabit gastrointestinal tract of humans, and animals (Tannock, 1990).The compositions of LAB in normal human and animal gastrointestinal flora are high (Sgouras *et al.*, 2004).The LAB composition differs significantly in environmental factors. Studies have reported that in infants and adults major autochthonous *Lactobacilli* species found are *L. ruminis*, *L. salivaris*, *L. reuteri* and *L. gasseri* (Reuter, 2001). In a study done by Bello and associates (2002) food associated LAB such as *L. sakei* and *L. mesenteroides* were found as intestinal inhabitants depending on the alternate incubation conditions of 30°C and 2% O_2 (Dal Bello *et al.*, 2003).

The differences in individual LAB composition is mostly in species level, probably due to antibiotic intake, diet difference and environmental factors (Donohue, 2004; Dicksved *et al.*, 2007). The most leading *Lactobacilli* found are *Lb. ruminis, Lb. reuteri/fermentum, Lb. salivarius* and *Lb. acidophilus/delbrueckii* (Naito *et al.*, 1995; Leser *et al.*, 2002; Yin and Zheng, 2005; Yun *et al.*, 2009).In humans LAB are more intense in the small and large intestine, also *Bifidobacterium* has been found to outnumber *Lactobacilli* (Finegold *et al.*, 1983).

Enterococci are also commonly found in the gut of human and animals (Vankerckhoven *et al.*, 2004). They are facultative-anaerobic gram positive, spherical in shape and occur singly, in pairs or in chains (Ciftci *et al.*, 2009). It is assumed that they can enter the environment by human and animal faeces (Kuhn *et al.*, 2000). *Enterococcus* sp are generally non virulent and cause little threat to human, but may be involved in nosocomial opportunistic infections due to resistance towards antimicrobial agents (Chenoweth and Schaberg, 1990).

The use of LAB in production of fermented foods and beverages has a long and safe history (Caplice and Fitzgerald, 1999; Leroy and Vuyst, 2004; Wood and Holzapfel, 1995). In a state like Sikkim, the estimated per capita consumption of ethnic fermented foods and beverages is 163.8 g/day, comprising 12.6% of total food intake (Tamang *et al.*, 2007).

2.3 Beneficial effect of LAB on human health

Presence of LAB as a normal microflora of human intestine has beneficial effects with is contribution to human physiology, particularly helps in digestion and minimises disorders caused by other pathogenic microbes (Toomey *et al.*, 2010). As they have been considered as an effective probiotics, as such it is a subject of discussion to know their potentiality to acquire and transfer genes related to antibiotic resistance (Liu *et al.*, 2009).

Particularly, the *Lactobacillus* species in the GIT have received remarkable attention due to their health-promoting actions (Walter, 2008).

The beneficial effects of LAB was first studied by the Russian scientist, E. Metchnikoff (1845 -1919), he proposed that the extended life span of the Balkan people was possibility due to their practice of taking fermented milk products (Metchnikoff, 1908). He assumed that disturbances in GIT could occur by growth of infectious microbes, as such LAB has a potential to prevent or minimise harmful effect of such harmful microbes.

LAB has been believed to posses numerous beneficial properties than other. Still when comes to role of LAB within the gastrointestinal tract, it remains a subject of controversy for the area of intestinal microbial ecology as less attempts are made to confirm that LAB improve the health of the host (Hove *et al.*, 1999).

The *Lactobacillus* species commonly used as probiotics includes *L. acidophilus, L. reuteri, L. casei, L. rhamnosus and L. delbruekii* (the starter culture for yoghurt) (Korhonen *et al.*, 2008). Nevertheless, with consideration to the taxonomic complexity of this microbial genus, still agreement seems to be lacking on the resistance or susceptibility for most antibiotics (Gueimonde *et al.*, 2013).The genus *Bifidobacterium* used as probiotics are from human origin.

2. 4 Antibiotic targets

Antibiotics are antimicrobial substances which hinder the process necessary for growth and survival of bacteria, but harmless to the host. There are three important targets of the antibiotics: i) cell-wall synthesis (e.g. betalactams, glycopeptides); ii) protein synthesis (e.g. macrolides, aminoglycosides and tetracyclines); iii) DNA replication and repair (e.g. fluoroquinolones) (Walsh, 2000).

2.5 Mechanism of antibiotic resistance

There are four major approach by which bacteria becomes resistance to antimicrobial agents: i) alteration of cell permeability by which the intracellular antibiotic concentration is decreased or ii) efflux mechanism; iii) antibiotic enzymatic inactivation; and iv) antibiotic target modification.

2.5.1 Intrinsic or acquired resistance

There are several mechanisms by which antibiotic resistance genes can be spreaded among bacteria. Antibiotic resistance in bacterial species could be either inherent referred as intrinsic or natural and acquired (Courvalin, 2006). Intrinsic or natural resistance is an inherent attribute of bacteria, which can be due to lowered permeability of antibiotics across the cell wall, lack of antibiotic site or low affinity presence of target site. In inherited resistance the resistance remains almost same for all bacterial strains of particular species, whereas in acquired resistance usually strains of susceptible species develops resistance to antimicrobial agent (European Commission, 2008). An example of intrinsic resistance is presented by heterofermentative *Lactobacilli*, caused by termination of dipeptides with D-lactate in place of D-alanine, which is the cell wall target site for glycopeptides (Billot-Klein *et al.*, 1994; Klein *et al.*, 2000). The bacterial strain can show resistance by acquiring resistance genes by mutation or by taking resistance genes via horizontal gene transfer.

Intrinsic resistance pose less threat of risk for horizontal spread. The minimum potential for horizontal transfer within the bacterial species is reported to be intrinsic resistance, as given in an example with presence of chromosomal resistant determinant for vancomycin of *Lactobacillus rhamnosus* strain CG (Tynkkynen *et al.*, 1998).

The best characterised intrinsic resistance to LAB is the resistance to vancomycin, with regards to other antibiotics. Vancomycin being the antimicrobial agent that targets bacterial protein synthesis at ribosomal level, when it comes to contact with peptidoglycan precursors it binds to the D-alanine terminus of pentapeptide, which results in prevention of polymerisation of peptidoglycan precursors. However, in several species of LAB, D-lactate or D-serine composition of muramyl pentapeptide replaces the D-alanine terminal residue, preventing binding of vancomycin (Delcour *et al.*, 1999) and shows antibiotic resistance

However, disparity between intrinsic and acquired resistance exists, the latter is considered to have higher potential for horizontal spread of antibiotic resistance, when resistance genes are present in plasmids and transposons, the mobile genetic elements (Khachatourians, 1998; European Commission, 2008). The intrinsic resistance to aminoglycosides is caused due to the inability of antibiotic uptake as cytochrome-mediated electron transport is absent (Charteris *et al.*, 2007).

LAB frequently possesses plasmids of different sizes, the antibiotic resistance determinants in plasmids have been reported in *Lactococcus lactis* and various *Lactobacillus* and *Enterococcus* species (Gevers *et al.*, 2003). Successively, many LAB are found to be resistant to antibiotics (Lavanya *et al.*, 2011). Apart from that intrinsically antibiotic LAB (probiotic) strains might have beneficial effects on patients whose normal intestinal flora has become disturbed or unbalanced due to excessive use of antimicrobial agents (Sanders and Huisint, 1999).

2.6 Food as a source of transmission of antibiotic resistant bacteria

Studies have confirmed that only in worst condition with high intake of food products containing resistant bacteria, horizontal transfer may occur (Jacobsen *et al.*, 2007). LAB, found in a variety of fermented food products as such have found to act highly as a reservoir (Zonenschain *et al.*, 2009). According to various studies food is considered to be one of the chief routes for transmission of antibiotic resistant bacteria among animal and humans. The chief point to be noted is that the intestinal bacteria might interact with the commensal bacteria while passing through the colon. This

factor could allow these bacteria to transmit antibiotic resistance genes (Salvers *et al.*, 2004).

The two facts that are the genetic basis of development of antibiotic resistance particularly derived from food are firstly the concerned bacteria must have come in contact with the antimicrobial substance, secondly, the pressure created by the antimicrobial agent force development of antibiotic resistance (Khachatourians 1998; Levy and Marshal, 2004). It has been reported that bacteria used as starter cultures for food production might contain antibiotic resistance genes (Danielsen and Wind 2003; Teuber, 1999).

Resistance to antibacterial substance tetracycline has been observed more frequently among *Lactobacillus* species, with broad range of MICs (Korhonen *et al.* 2008). Also, *Lactobacilli* very often are resistant to aminoglycosides such as neomycin, kanamycin, streptomycin, and gentamycin (Danielsen and Wind 2003, Coppola *et al.*, 2005, Zhou *et al.*, 2005).The antimicrobials based on mode of action are divided into several groups. Currently, more than 250 antibiotics exists for therapeutic purpose of which more than 100 are β- lactams, that targets specific sites on bacteria (van den Bogaard and Stobberingh 1996). The targeting sites of these antimicrobial drugs comprise cell wall synthesis, protein synthesis, DNA gyrase folic acid metabolism (Neu, 1992). In various studies genes regulating resistance located on transferable elements (plasmids and transposons) to several antimicrobial substances such as erythromycin, streptomycin, chloramphenicol, vancomycin and streptomycin has been characterised in Lactobacilli (Gfeller*et al.*, 2003) and *Lactococci* (Perreten *et al.*, 1997) from food substances.

2.7 General overview of susceptibility patterns in LAB

It was apparent that *Lactobacilli* with regards to specific antibiotics, generally shows great sensitivity to antibiotics such as penicillin and β-lactamase that targets cell wall but are resistant to cephalosporins. It has been studied that many *Lactobacillus* species (Gueimonde *et al.*, 2013) and *Enterococci* shows resistance highly to vancomycin. The resistance in *Enterococci* is believed to have achieved with the presence of nucleotide sequences related to vanA (Dutka-Malen *et al.*, 1990), vanB (Hayden *et al.*, 1993) and vanC (Quintiliani *et al.*, 1993).

Some *lactobacillus* sp. preferably *L. casei*, *L. plantarum*, *L. rhamnosus* and several *Enterococci* strains isolated from clinical samples have shown transferable vancomycin resistance (Cooper *et al.*, 1998; Shlaes *et al.*, 1989). Thus it is a clear

indication that such cases must have involved antibiotic medication (Shlaes *et al.*, 1989; Witte, 1998). Sensitivity to penicillins is shown greatly by *Lactobacilli* (Danielsen and Wind, 2003; Goldstein *et al.*, 2000) as well as low sensitivity to ampicillin is also observed (Goldstein *et al.*, 2000).

Majority of *Lactobacilli* tend to show less inhibitory effect on inhibitors of nucleic acid synthesis whereas, for many protein inhibitors such as chloramphenicol, macrolides, tetracycline and lincosamides, *Lactobacilli* show high susceptibility even in low concentrations. It is generally resistance to aminoglycosides, whereas resistance to other antibiotics greatly differ (Gueimonde *et al.*, 2013).

In a study done by Roberts in 2008, it was found that approximately 66 genes accounts for bacteria acquiring resistance to antibiotics microlide, lincosamide, and streptogramin (MLS) by three mechanisms. The resistance is acquired by a directly modifying the drug targets, occasionally by inactivating the antibiotics itself and also by efflux mechanism (Roberts, 2005). While considering the efflux pump, genes involved in the efflux activity of Gram-positive bacteria includes *mef*A, *mef*E, *mrs*A, *and mre*A (Rao and Halami, 2012). In Gram positive bacteria, the gene *mrs*A, regarded as a member of the ATP- binding cassette, one of the transporters, provides resistance to erythromycin and type B streptogramins (Reynolds *et al.*, 2003). Commonly, LAB shows susceptibility to antibiotics such as erythromycin and tetracycline, which targets protein synthesis (Ammor *et al.*, 2007).

A group of broad spectrum antibiotics Tetracycline has a wide spread use for a treatment against Gram positive and Gram negative bacteria. Currently, 39 acquired tetracycline resistant (TC^r) determinants have been reported for bacteria (Roberts, 2005). These set of genes is responsible for energy dependent efflux mechanisms for proteins, which provide protections to bacterial ribosome. Rarely, direct inactivation of antibiotics occurs by mutating 16S rRNA gene, which guards the ribosome from tetracycline (Roberts, 2005).

As evident, LAB can survive and grow in various food materials and human colon; suggestions have been made that subpopulations of certain LAB could at as reservoir and carrier of antibiotic resistant genes within the human body (Teuber *et al.*, 1999; Gevers *et al.*, 2000).

2.8 Identification of AST in LAB

The lowest concentration of antibiotics that inhibits the visible bacterial growth is known as the minimal inhibitory concentration (Phillips *et al.*, 1991; Piddock, 1999). MIC is expressed as µg/ml of antibiotic. Another commonly used method is agar diffusion test standardised by Bauer *et al.*, 1996. In this method antibiotic discs are used, sensitivity of antibiotics towards the antibiotics is determined by zone of inhibition (Bauer *et al.*, 1996). This method was recommended mainly for fast growing pathogens such as *Staphylococcus* sp and *Enterobacteriaceae*, the same results cannot be merely interpreted to the genera belonging to *lactobacillus*. Hence, study on antibiotic susceptibility testing with disc diffusion, with refinements for species of *Lactobacillius* was published (Charteris *et al.*, 1998).

Various methods has been reported to determine MIC for LAB that belongs to genera *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Bifidobacterium* based on different methods such as agar disk diffusion (Charteris *et al.*, 1998; Yazid *et al.*, 2000) E- test (Charteris *et al.*, 2001); Croco and Erwin *et al.*,1994) agar dilution (Katla *et al.*, 2001) and broth dilution (Yamane *et al* ., 1991). The conventional methods such as Muller- Hinton and Iso- Sensitest (IST) agar or broth could not be considered as suitable medium for these organisms, as they need special growth conditions in regards to acidity of medium and nutrient supplementation. Therefore, to perform AST of LAB, specific medium which is referred as LAB susceptibility test medium (LSM) with or without additional supplement of L-cysteine has been developed (Klare *et al.*, 2005).

The new growth medium known as LSM was developed principally to maintain the growth of *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Bifidobacteria,* without having any sort of antagonistic interactions between components of the medium and specific antimicrobial agents (Klare *et al.*, 2005). Several studies have also been done on phenotypic antibiotic resistance of LAB using LSM broth and agar (Klare *et al.*, 2007; Devirgiliis *et al.*, 2008; Huys *et al.*, 2008).

2.9 Role of GUT microbiota

Gastrointestinal tract (GIT) of vertebrate, including that of humans, is habitat to an enormous group of microbiota, mainly bacterial, species, referred to as the gut microbiota. On comparisons of the characteristics of germ-free animals and those of conventional animals, the demonstration is clear that the gut microbiota has significant influence on host immunology, biochemistry, physiology, and provides necessary resistance to gut infections (Berg, 1996; Gordon and Pesti, 1971).

The microorganisms tend to colonise different sites of gastrointestinal tract (GI) with a difference on the different physiological condition of the GI such as pH, nutrient availability, flushing action of the food material, presence of antimicrobial peptides, immunoglobulin, bile salts, etc (Kanno *et al.*, 2009; Hapfelmeier *et al.*, 2010. The GIT is, fundamentally, a continuous tube extending from the mouth to the anus. The regions of the GIT include the oral cavity, the oropharynx, and laryngopharynx, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), and the large intestine (caecum, colon, and rectum). Since microorganisms are diverse in their anatomy, physiology, organization, and location, each of these regions provide a different set of environmental conditions for potential microbes to colonize their favorable sites. Thus, human gut microbiota as a whole has 150 times more genes in their collective metagenome than are present in the human host genome (Qin *et al.*, 2010).

Study reports explains that the normal gut structure and functioning results from a set of complex interactions within the host and microbes in the gut. In a study, germ-free mice was colonized by using *Bacteroides thetaiotaomicron*, it leads to a conclusion that, this commensal showed influence in many genes related to nutrient absorption, mucosal barrier function, xenobiotic metabolism, angiogenesis, the enteric nervous system and postnatal intestinal maturation (Midtvedt, 1999). Due to gene diversity, though there is dissimilarity in biochemical pathways between host and microbial community that resides in the host, it successfully provides different enzymes to the host. In the colon the major source of energy remains fermentation of carbohydrate. As such, the colonic microorganisms support vitamin synthesis and absorption of calcium, magnesium, and iron (Roberfroid *et al.*, 1995; Miyazawa *et al.*, 1996; Younes *et al.*, 2001).

Therefore, other than sugar fermentations, they have evolved electrogenic decarboxylation and ATP-forming deamination. The correct balance between sugar fermentation and decarboxylation favours buffered environment in the gut for their survival in human gastric trait and colonisation. A cross talk between LAB and hosts exists. Gut stimuli enhances production of moonlight proteins that leads to adhesion of bacteria to mucosa which stimulates the immune cells. Subsequently, LAB shows antagonistic effect that makes it anti- infective to other microorganisms (Pessione, 2012).

Various study supports the importance of microbiota in health and disease, where a slight changes can drastically link to inflammatory and metabolic disorders (O'Toole and Claesson, 2010), as well as inflammatory bowel disease (Frank *et al.*, 2007; Dicksved *et al.*, 2008; Qin *et al.*, 2010), irritable bowel syndrome (Kassinen *et al.*, 2007; Jeffery *et al.*, 2012); neurological disorders (Gonzalez *et al.*, 2011) and cancer (Lupton, 2004), obesity (Ley *et al.*, 2006) in adults, malnutrition (Kau *et al.*, 2011) and weakness (Van Tongeren, 2005). According to the study done by Claesson *et al.*, (2012) on 178 older individuals it was observed that the microbiota of older people varies from that of younger individuals, with a slight inter individual difference. The study also indicates a relationship between diet, microbiota and health status, and indicate a role for diet-driven microbiota alterations with the progression of age of the individuals.

The microbiota is essential for homeostasis in the intestine (Garrett *et al.*, 2010) and chronic activation of the innate and adaptive immune system is linked to immunosenescence (Guigoz *et al.*, 2008). Several correlations have been made in the past between specific components of the microbiota and pro-inflammatory cytokine levels, but these did not separate young adults from older people, the LAB content in humans usually do not show much differences (Biagi *et al.*, 2010).

Chapter - IV

Materials and Methods

4. Materials and Methods

4.1. Materials

4.1.1. Culture Media

4.1.2. Arginine Hydrolysis Medium (Thornley, 1960)

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

4.1.3. Carbohydrate Fermentation Medium (Schillinger and Lücke, 1987)

Peptone	10 g
Yeast extract	5 g
Potassium phosphate	2 g
Tri-sodium citrate	2 g
Carbohydrate	0.5 %
Tween 80	1 ml
Sodium acetate	5 g
Magnesium sulphate	0.58 g
Manganese sulphate	0.28 g
Phenol red	0.004 %
Distilled water	1000 ml

4.1.4. Hi- sensitivity Test Agar (M485 Hi Media Mumbai) (Klare *et al.*, 2007)

Casein enzymic hydrolysate	11.00g
Peptic digest of animal tissue	3.00 g
Dextrose	2.00 g
Sodium chloride	3.00 g
Starch, soluble	1.00
Disodium phosphate	2.00 g
Sodium acetate	1.00 g
Magnesium glycerophosphate	0.02 g
Calcium gluconate	0.10 g
Cobaltous sulphate	0.001 g
Cupric sulphate	0.001 g
Zinc sulphate	0.001 g
Ferrous sulphate	0.001 g
Manganous chloride	0.002 g
Menadione	0.001 g
Cyanocobalamin	0.001 g
L-Cysteine hydrochloride	0.02 g
L- Tryptophan	0.02 g
Pyridoxine hydrochloride	0.003 g
Calcium pentothenate	0.003 g
Nicotinamde	0.003 g
Biotin	0.0003 g
Thiamine hydrochloride	0.00004 g
Adenine	0.01 g
Guanine	0.01 g
Xanthine	0.01 g
Uracil	0.01 g
Agar	8.00 g

4.1.5. LAB Susceptibility Medium

The LAB isolates were tested for different antibiotics following the modified standard Kirby – Bauer procedure as used by (Klare *et al.*,2007) on LAB susceptibility test medium (LSM) (90% MRS Agar,10% Iso- sensitest Agar) as developed by (Klare *et al.*, 2005).

4.1.6. MRS Agar (M641, HiMedia, Mumbai) (de Man, Rogosa, Sharpe *et al.*, 1960)

Peptone	10 g
Beef extract	10 g
Yeast extract	5 g
Potassium phosphate	2 g
Tri-sodium citrate	2 g
Dextrose	20 g
Tween 80	1 ml
Sodium acetate	5 g
Magnesium sulphate	0.58 g
Manganese sulphate	0.28 g
Calcium carbonate	1.5 %
Agar	20 g
Distilled water	1000 ml

4.1.7. MRS Broth (M369, HiMedia, Mumbai)

(Includes all the above ingredients excluding Agar)

4.1.8. Muller Hinton Agar (Cappuccino, Sherman, 2007)

Muller Hinton Agar	38gm
NaCl	2%
Distilled water	1000 ml

Used to perform antimicrobial susceptibility test for control organisms.

4.1.9. Nutrient Broth (Cappuccino, Sherman, 2007)

Peptone	5g
Nacl	5g
Beef Extract	3g
Distilled water	1000ml

4.1.10. Nutrient agar (Cappuccino, Sherman, 2007)

Peptone	5g
Nacl	5g
Beef Extract	3g
Agar	15 g
Distilled water	1000ml

Used for subculturing and maintenance of the control strains, *Escherichia coli* MTCC 1089 and *Staphylococcus aureus* MTCC 7443.

4.2. Reagents

4.2.1. Burke's Iodine Solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

4.2. 2. Gram's Crystal Violet (S012, HiMedia, Mumbai)

4.2.3. Glaciel acetic acid

4.2 4. Nessler's Reagent

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

Potassium iodide was dissolved in 35 ml of distilled water followed by addition of saturated aqueous solution of mercuric chloride till the appearance of precipitate.

Then, 400 ml of potassium hydroxide was added and made the final volume to 1000 ml by adding distilled water. The solution was left for a week; the supernatant was decanted and stored in capped amber bottle at 4° C.

4.2 5. Safranin (S027, HiMedia, Mumbai)

4.2.6. Mac Farland 0.5 (R092-INO, HiMedia, Mumbai)

4.3. Antibiotics

4.3.1. Antibiotic discs

Ampicillin (10 mcg) (SD002), Amikacin (SD035), Clindamycin (SD164), Chloramphenicol (SD006), Erythromycin (SD013), Kanamycin (SD223), Methicillin (SD019), Norfloxacine (SD057) Penicillin G (SD028), Streptomycin (SD031), Tetracycline (SD037), Vancomycin (SD045) (commercial discs from Himedia).

4.3.2. Antibiotics powder

Streptomycin (CMS220-5G), Vancomycin (VANKING 500mg), Chloramphenicol (CMS218-5G).

4.4. Test organisms

Bacteria	**Obtained from**
Gram negative *Escherichia coli*	MTCC, Chandigarh (MTCC 1089)
Gram positive *Staphylococcus aureus*	MTCC, Chandigarh (MTCC 7443)

4.5. Methodology

4.5.1. Field Survey

The field survey was conducted on East district of Sikkim. The survey was based on the structured questionnaire (**Annexure I**). The questionnaire was designed as such to identify the individuals who were healthy enough to have taken antibiotics rarely (once or twice) and those who have not directly consumed antibiotics in their life time. These individuals have been using medicinal plant sources for treating minor illness. They have claimed to have never been hospitalised and has an active lifestyle. The questionnaire was designed from all aspects to make positive that they were living actually healthy.

The questionnaire included history of disease that they had suffered and hospitalisation, if any. The subject's demographics (gender, age), socio-economic (e.g. profession), dietary habits (e.g. vegetarian/non-vegetarian, fermented/non-fermented foods), physical activity, life-style behaviour (e.g. addictions like smoking and alcohol consumption) and medical history (e.g. diseases, medications) were also taken into consideration for the study.

The information regarding the study was provided to the participants before. The questionnaire was handed over with the approval of the participants (**Annexure II**). Names and information drawn during the study were kept confidential.

4.5.2. Collection of samples

Faecal samples were collected from healthy individuals who claimed to have taken medicines or antibiotics rarely and those who claimed to have never consumed allopathic medicines but traditional medicines for minor illness. The samples were collected in sterile stool collection container and were transported to the laboratory in a mini cooler. The samples were stored at 4-8° C for analysis. All the samples were analysed on the same day of collection. However, only 6 valid samples were finally analysed.

4.5.3. Isolation

2 g of sample was dissolved in 18 ml physiological saline (0.1%) and homogenized in stomacher lab-blender (400, Seward, UK) 230 rpm for 10-12 minutes. Serial

oil-immersion objective. The specimen was examined and compared with both negative and positive control (Bartholomew, 1962).

4.5.5.3. Motility test

24 hour cultures were inoculated into SIM Agar tubes using the stab inoculation technique and incubated at 37° C for 24- 48 hours. If the culture is motile growth will be seen to move away from the stab and into the surrounding medium (Harrigan *et al.*, 1976)

4.5.5.4. Potassium Hydroxide Test

Potassium hydroxide (KOH) test was performed following the method of Halebian *et al.*, (1981).The test was performed by placing 3% KOH solution on microscope slide followed by addition of generous amount of 24 hour culture to the drop of KOH with an inoculating loop. The KOH culture mixture was stirred in circular motion with the loop and occasionally raising the loop. The positive result was indicated with the presence of mucoid or viscous appearance while raising the loop. Absence of mucoid or viscous appearance and no string of mixture indicated negative result. Gram positive bacteria are negative for KOH test while Gram negative bacteria are positive for this test.

4.5.5.5. Catalase Production

The production of gas bubbles were observed by adding 0.5 ml of 10 % hydrogen peroxide solution (Merck) to the cultures as described by Schillinger and Lücke, 1987. The production of gas bubbles indicates the presence of catalase, while absence of gas bubbles indicated absence of catalase.

4.5.5.6. Gas (CO_2) production from glucose

CO_2 production from glucose is the major criterion for the determination of homo-fermentative or hetero-fermentative nature of an LAB isolates. Tubes of 10 ml MRS broth without citrate and containing inverted Durham tubes was inoculated with 24 hour-old cultures and incubated at 30°C (Schillinger and Lücke, 1987). Accumulation of gas in the inverted Durham tubes indicated positive result whereas; no gas production indicates negative result.

4.5.5.7. Ammonia from Arginine

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

4.5.5.8. 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 broth was filter sterilized, added 5ml to each tube and inoculated with 24 hour-old MRS broth culture. The tubes were incubated at 30° C for 24-72 hour and observed for the growth (Dykes *et al.*, 1994).

4.5.5.9. Growth at different temperatures

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

4.5.5.10. Growth in different NaCl concentrations

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

4.5.5.11. Acid from carbohydrates

LAB isolates were screened for their ability to ferment 6 different carbohydrates. The method based on Schillinger and Lücke (1987). Tubes of 4 ml carbohydrate fermentation broth (MRS broth without beef extract, containing 0.5% w/v of different carbohydrates instead of glucose and 0.004% phenol red indicator) were inoculated and incubated at 30° C for 2-5 days. Colour change from red to yellow indicated acid production.

4.5.5.12. Phenotypic identification

Bacterial species were identified following the taxonomic keys of Bergey's Manual (Sneath *et al.*, 1986), The Prokaryotes (Dworkin *et al.*, 2006); (Simpson and Taguchi, 1995); (Wood and Holzapfel, 1995).

4.5.6. Antimicrobial Susceptibility Testing

4.5.6.1. Preparation of test organisms

The test organism for the study includes one genus of Gram negative bacteria, *Escherichia coli* and one genus of Gram positive bacteria *Staphylococcus aureus*. The test organisms were maintained at 4°C on a nutrient agar slants. Active cultures for experiments were prepared by transferring loopful of culture in a flask containing nutrient broth, and were incubated at 37°C for 24 hrs.

4.5.6.2. Preparation of bacterial suspension

For each organism three to five isolated colonies were picked from fresh agar plate and selected colonies were transferred into a tube containing 3-4ml of suitable nutrient broth medium. After proper mixing, the broth cultures were incubated at 37°C for 24 hrs. Turbidity was assessed by Spectrophotomter (Eppendorf make Biophometer) by measuring the absorbance of the suspension. The absorbance should be in the range of 0.08-0.13 OD at 625 nm equivalent to McFarland standard 0.5. Turbidity was adjusted by adding sterile saline, when the turbidity was higher than required and by adding bacterial colony when the turbidity was too low and further incubating (Wiegand *et al.*, 2008).

4.5.6.3. 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°C for 24 hours. Turbidity of bacterial cultures was adjusted to 0.5 McFarland standards, equivalent to cell density of (10^8 CFU/ml). The bacterial lawn was made on LSM Agar using sterile cotton swab. The streaking was done on the entire surface of the LSM agar plates thrice; the plate was turned 60 degree between streaking to achieve uniform inoculation. The plates were allowed to dry for 10-15 minutes. Using sterile forceps the antibiotics discs were placed on the surface of LSM agar aseptically.

Incubation was done at 37°C for 24-48 hours under microaerophilic condition using candle jar.

After proper incubation the diameter of zone of inhibition was measured using Hi media scale results were expressed as sensitive (S; ≥ 21mm), intermediate (I; 16-20 mm) and resistant (R; ≤ 15 mm) (Charteris *et al.*, 1998, Volkova *et al.*, 2006), with some exceptions; zone ≤ 19mm for penicillin G is considered resistant, zone ≤ 14mm for tetracycline and vancomycin, zone ≤ 13 mm for kanamycin and chloramphenicol are considered resistant (Charteris *et al.*, 1998).

The test was done with *Escherichia coli* MTCC 1089 and *Staphylococcus aureus* MTCC 7443 as control strain. The results obtained were compared to these two strains as published by the CLSI, (2012).

4.5.6.4. Minimum inhibitory concentration (MIC) by agar dilution method

Minimum inhibitory concentration (MIC) was performed to evaluate the phenotypic antimicrobial resistance of a strain to a certain antibiotic following the method of Korhonen *et al.* (2010). The MIC was defined as the smallest amount of antibiotic needed to totally inhibit the growth of the bacteria after incubation for 48 h. For performing MIC, single pure colony was inoculated in a sterile saline (0.9%), the turbidity was matched to McFarland standard 0.5equivalent to 10^8 CFU/ml, and the cell density was also checked in spectrophotometer (Brand) with an OD_{625} of 0.16-0.20.

The 1µl aliquots of each bacterial suspension were spotted on the LSM agar previously mixed with antibiotics. The serial two-fold dilutions of the antibiotic were prepared mixed with the LSM agar prior to plating, using a wide range of variable concentrations depending on the antibiotic and incubated. For Minimum Inhibitory Concentration three antibiotics were used streptomycin, chloramphenicol and vancomycin. The solvent used to dissolve vancomycin and streptomycin was water as water is the suitable solvent. The solvent used for chloramphenicol was 95% glacial acetic acid.

Chapter - V

Results

5. Results

The field study was conducted prior to sample collection, to identify individuals who were "rare user of antibiotics" and those who were "frequent traditional medicine user" based on the prepared questionnaire (**Annexure I**). Rare antibiotic user group includes those individuals who claimed that they had taken antibiotics only few times. Frequent traditional medicine user group of individuals claimed that they prefer traditional medicine for any kind of ailments and had never taken any allopathic medicines including antibiotics. With the help of the questionnaire the previous history of illness and medication were cross-checked. Thus these individuals were healthy with no history of chronic infection and had an active lifestyle.

In total, 25 individuals were surveyed out of which only 6 individuals were considered for sample collection. The age groups of individual subject ranged from 25-45 years, 02 participants fell under the age group 25-35, while 04 individuals belong to 35-45 age groups as shown in (Table 1). Finally, after the valid entries, 03 participants were male and the 03 participants were female.

In our study, 03 participants preferred traditional medicines, 03 individuals rarely consumed allopathic medicine along with traditional medicine. The people who takes traditional medicine has a belief that these medicine has no side effects and are easily available than modern medicine.

Every individual were found to consume traditional fermented foods, mainly gundruk, sinki churpi, curd (dahi) and kinema, which are part of their daily diet. Some also preferred traditional fermented beverages. Traditional fermented foods are a vital part of the diet of the people of Sikkim (Tamang, 2007).

5.1 Microbial analysis

The samples were processed and after proper incubation of 48-72 hours at 37°C, the colonies were counted using the colony counter pen (Cole Parmer). The colony forming units of sample per gram was calculated and converted to log CFU/g. The microbial load of LAB was calculated considering various factors as discussed above. According to the calculation done, the microbial load of age group 25-45 and 35-45 was 10.33±0.57 and 9.66±0.57 respectively, Table 1. Subsequently, the microbial load of different food habits (vegetarian, non-vegetarian) and medication preferred (allopathic, traditional) are 9.5±0.70, 10.25±0.70, 9.66±0.81 and 10.33±0.70 respectively as given below in (Table 1).

Table 1: Microbial load of stool sample in various categories of individuals.

Categories	Place of collection	Microbial load (log CFU/g)
Age group		
25-35 (n=2)	n=1 (Aritar, East Sikkim) n=1 (Arithang, East Sikkim)	10.33±0.57
35-45 (n=4)	n=1 (Aritar, East Sikkim) n=3 (Arithang, East Sikkim)	9.66±0.57
Food Habits		
Vegetarian (n=2)	n=1 (Arithang, East Sikkim) n=1 (Aritar, East Sikkim)	10.25±0.70
Non vegetarian (n=4)	n=1 (Aritar, East Sikkim) n=3 (Arithang, East Sikkim)	9.5±0.70
Medication practiced		
Frequent traditional medicine user (n=3)	n=2 (Aritar, East Sikkim) n=1 (Arithang, East Sikkim)	10.33±0.70
Frequent antibiotic user (n=3)	n=3 Arithang, East Sikkim)	9.66±0.81

Note=*SD* (+/-) Standard Deviations value is included along the log Colony Forming Units. n, number of samples.

5.2. Characterization of LAB Isolates

A total of 126 lactic acid bacterial isolates were isolated. Specific codes were assigned to the isolates (*H=Human, F=Feces, Ar/At= Aritar / Arithang (name of places), 1, 2, 3 etc = sample number, a,b,c,A,B,C etc.= alphabetical sequence* for the isolates, *e.g.; HFAr:1a, HFAt:6a.* Hence, 62 isolates from rare antibiotic users and64 from frequent traditional medicine users were isolated.

The isolates showed variation in colony morphology on MRS agar as given in (Table 2, 3). Many were circular, large, elevated, thick milky white and elevated colonies. Some were small elevated, circular, pale white colonies. Some were large or small pale yellowish elevated colonies, some were small, shiny, pale white circular colonies and some were medium-sized, pale white colonies.

The cell morphology of the isolates was observed under 100x oil immersion objective after staining with saffranin. The cell morphology observed were ovoid, coccus in tetrad, cocci arranged singly and in chains, cocci in pairs. The rods observed were with rounded ends, thin rods, rods in chains, clusters and some tiny rods. Since, all the isolates were gram positive, non-motile and catalase negative, they were taken for further identification tests. None of the isolates showed gas production. Therefore, all the isolates were homofermentative LAB.

For the growth at different pH 19 isolates (HFAr:6c , HFAr:6j , HFAr:6J, HFAr:6K, HFAt:4c, HFAt:3g, HFAt:3a , HFAt:4e, HFAt:4g, HFAt:4i, HFAt:4q, HFAt:4v, HFAt:4w, HFAt:4x , HFAt:4A, HFAt:5e, HFAt:5h , HFAt:5s and HFAt:5t showed growth at pH 3.9 and 8 isolates (HFAr:6g, HFAr:6h, HFAr:6i, HFAr:6m , HFAr:6r HFAr:6l, HFAr:6r, HFAr:6v and HFAt:5b) were weakly positive, rest of the isolates showed no growth. At pH 9.6, 20 isolates (HFAt:2f, HFAr:6a, HFAr:6c, HFAr:6g, HFAr:6h, HFAr:6i, HFAr:6l, HFAr:6m, HFAr:6r, HFAr:6s, HFAr:6v, HFAt:3f, HFAt:4e, HFAt:4g, HFAt:4i, HFAt:4p, HFAt:4v, HFAt:4w, HFAt:5l and HFAt:5q) showed no growth while other 106 isolates showed growth (Table 2, 3).

All the isolates showed growth at NaCl concentration 6.5% but no growth was observed at NaCl concentrations 10% and 18% (Table 2, 3). The majority of the isolates 67% were able to produce ammonia from arginine and 33% could not produce ammonia from arginine (Table 2, 3).

5.3. Fermentation of carbohydrate

Carbohydrate fermentation is an important test for characterisation of bacteria. The isolates were subjected to test for six sugars (Galactose, Maltose, Raffinose, Rhamnose, Mannitol, and Sucrose) (Table 2, 3).

Almost all the isolate show similar results for most sugars with differences in fermentation pattern of few sugars. The sugars galactose and rhamnose was fermented by all the isolates. Maltose fermentation was observed in all the isolates except, HFAt:3o, HFAt:4j and HFAr:6E.

The fermentation of raffinose was observed in all the isolates except HFAt:3a, HFAt:3e, HFAt:3h, HFAt:3i, HFAt:3l, HFAr:6m, HFAr:6n, HFAr:6p and HFAt:4b. The sugar sucrose were fermented by majority of the isolates except HFAt:1i, HFAt:1j, HFAt:3b,HFAr:6s, HFAr:6A, HFAr:6C, HFAr:6F, HFAr:6H, HFAr:6L and HFAr:6M. The isolates, HFAt:3o, HFAr:6N, HFAr:6p showed negative result for sugar mannitol.

5.4. Phenotypic Identification

Total 126 isolates of LAB were isolated, among these isolates, HFAt:1a, HFAt:1f, HFAt:1i, HFAt:1j, HFAt:1k, HFAt:2a, HFAt:2b, HFAt:2f, HFAt:2g, HFAr:6a, HFAr:6c, HFAr:6g, HFAr:6h, HFAr:6i, HFAr:6j, HFAr:6l, HFAr:6m, HFAr:6n, HFAr:6o, HFAr:6a, HFAr:6v, HFAr:6L, HFAr:6J, HFAr:6a, HFAt:3a, HFAt:3c, HFAt:3e, HFAt:3f, HFAt:3g, HFAt:3h, HFAt:3i, HFAt:3l, HFAt:4c, HFAt:4d, HFAt:4e, HFAt:4o, HFAt:4s, HFAt:3a HFAt:4v, HFAt:4v, HFAt:4w, HFAt:4x, HFAt:4y, HFAt:4z, HFAt:4B, HFAt:3a, HFAt:4C, HFAt:4D, HFAt:5i, HFAt:5l and HFAt:5q were identified as *Lactobacillus* sp (Table 2, 3).

The isolates HFAt:1b, HFAt:1c, HFAt:1d, HFAt:1e, HFAt:2d, HFAt:2e, HFAr:6e, HFAr:6u, HFAr:6B, HFAr:6D, HFAr:6G, HFAr:6K, HFAr:6O, HFAr:6P, HFAr:6Q, HFAr:6R, HFAt:3d, HFAt:3m, HFAt:4k, HFAt:4l, HFAt:4n, HFAt:4q, HFAt:4r,

HFAt:4A, HFAt:5a, HFAt:5b, HFAt:5c, HFAt:5d, HFAt:5e, HFAt:5g, HFAt:5h, HFAt:5l, HFAt:5m, HFAt:5n, HFAt:5p, HFAt:5s, HFAt:5u, HFAt:5v were identified as *Enterococcus* sp (Table 2, 3).

The isolates HFAt:1g, HFAt:1h, HFAt:2c, HFAt:2h, HFAt:2i, HFAt:2j, HFAt:2k, HFAr:6d, HFAr:6k, HFAr:6t, HFAt:5f, HFAt:5j, and HFAt:5o were identified as *Streptococcus* sp (Table 2, 3).

The isolates HFAr:6p, HFAr:6s, HFAr:6A, HFAr:6C, HFAr:6E, HFAr:6F, HFAr:6H, HFAr:6K, HFAr:6L, HFAr:6M, HFAr:6N, HFAt:3b, HFAt:3o, HFAt:4b, HFAt:4f, HFAt:4g, HFAt:4h, HFAt:4m, HFAt:4t, HFAt:4u, HFAt:5k and HFAt:5t were identified as *Pediococcus* sp (Table 2, 3).

For final identification all the previous tests done, including arginine hydrolysis, growth at different temperature and growth at different salt concentration was taken into consideration. After the tests done, majority of *Lactobacillus* species were positive for all the sugars, with some showing negative results for sucrose and raffinose. *Enterococcus* sp and *Streptococcus* sp was positive for all the tested sugars. *Pediococcus* sp showed negative result to sucrose, some to mannitol and some were maltose variable, but positive for rest of the sugars.

Table 2: Phenotypic characterisation of LAB isolated form rare antibiotic user.

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl conc.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:1a	Large yellowish shiny	Rod	–	+	–	+	–	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:1b	Small circular, pale white	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:1c	Small circular, pale white	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:1d	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:1e	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:1f	Small circular, milky white	Rod	–	+	–	+	–	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:1g	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus*sp
HFAt:1h	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAt:1i	Small circular, milky white	Rod	+	+	–	+	–	–	+	+	+	+	+	+	–	*Lactobacillus* sp
HFAt:1j	Large yellowish shiny	Rod	+	+	–	+	–	–	+	+	+	+	+	+	–	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed for all the isolates; +, positive results; –, negative results.

Table 2 (*Continued*)

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl conc.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:1k	Small circular, milky white	Rod	–	+	–	+	–	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:2a	Small circular, mucoid	Rod	–	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:2b	Small circular, milky white	Rod	–	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:2c	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAt:2d	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:2e	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:2f	Large yellowish, shiny	Rod	+	+	–	+	–	–	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:2g	Large circular, milky white	Rod	-	+	–	+	–	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:2h	Small circular, mucoid	Coccus	-	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAt:2i	Small circular, mucoid	Coccus	-	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C;growth was observed for all the isolates +, positive results; –, negative results.

Table 2 ***(Continued)***

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl con.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:2j	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAt:2k	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAr:6a	Large, elevated, milky	Rod	–	+	–	+	+	–	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6b	Small circular, milky	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6c	Large, elevated, milky	Rod	+	+	–	w+	+	+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6d	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAr:6e	Large circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6f	large, elevated, milky	Rod	–	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6g	Large, elevated, milky	Rod	+	+	–	+	+	w+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6h	Large, elevated, milky	Rod	-	+	–	+	+	w+	–	+	+	+	+	+	+	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed on all the isolates; +, positive results; –, negative results.

Table 2 ***(Continued)***

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl conc.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAr:6i	Small circular, milky	Rod	-	+	–	+	+	w+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6j	Large, elevated, milky	Rod	+	+	–	+	+	+	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6k	Small circular, mucoid	Coccus	–	+	–	w+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAr:6l	Small circular, milky	Rod	+	+	–	+	–	w+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6m	Small circular, milky	Rod	+	+	–	w+	+	w+	–	+	+	+	–	+	+	*Lactobacillus* sp
HFAr:6n	Large, elevated, milky	Rod	+	+	–	w+	+	–	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAr:6o	Large circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6p	Small circular, milky, shiny	C/T	+	+	–	+	+	–	+	+	+	–	–	+	+	*Pediococcus* sp
HFAr:6q	Small circular, mucoid	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6r	Small circular, milky	Rod	–	+	–	+	+	w+	–	+	+	+	+	+	+	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 2 (*Continued*)

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl conc.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAr:6s	Small circular, milky, shiny	C/T	–	+	–	+	+	–	–	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6t	Small circular, mucoid	Coccus	–	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAr:6u	Circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6v	Small elevated, milky	Rod	–	+	–	+	+	w+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6A	Large circular, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6B	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6C	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6D	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6E	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	–	+	+	+	*Pediococcus* sp
HFAr:6F	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6G	Large circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 2 ***(Continued)***

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl conc.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAr:6H	Small shiny, mucoid	C/T	–	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6I	Large circular, milky	Rod	–	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAr:6J	Small circular, milky	Rod	–	+	–	w+	+	+	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAr:6K	Small circular, pale white	Coccus	+	+	–	+	+	+	+	+	+	+	–	+	+	*Enterococcus* sp
HFAr:6L	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6M	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAr:6N	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	–	+	+	+	*Pediococcus* sp
HFAr:6O	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6P	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6Q	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAr:6R	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 3: Phenotypic identification of the LAB isolates of frequent traditional medicine users.

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl con.		Temp.		pH								
				6.5%	10%	10^0C	45^0C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:3a	Small circular, milky white	Rod	+	+	–	+	+	+	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAt:3b	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAt:3c	Small circular, milky white	Rod	–	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:3d	Large, mucoid, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:3e	Large circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAt:3f	Small circular, milky white	Rod	–	+	–	+	+	–	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:3g	Large circular, milky white	Rod	–	+	–	+	–	+	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:3h	Small circular, milky white	Rod	–	+	–	+	–	–	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAt:3g	Large circular, milky white	Rod	–	+	–	+	–	+	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:3h	Small circular, milky white	Rod	–	+	–	+	–	–	+	+	+	+	–	+	+	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 3*(Continued)*

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl con.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:3i	Small circular, milky white	Rod	–	+	–	+	–	–	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAt:3l	Large circular, milky white	Rod	–	+	–	+	+	–	+	+	+	+	–	+	+	*Lactobacillus* sp
HFAt:3m	Large, mucoid, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:3o	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	–	+	+	+	*Pediococcus* sp
HFAt:4a	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4b	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	–	+	–	*Pediococcus* sp
HFAt:4c	Small circular, milky white	Rod	–	+	–	+	+	+	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4d	Large circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4e	Large circular, milky white	Rod	+	+	–	+	+	+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4f	Small shiny, mucoid	C/T	–	+	–	+	+	–	+	+	+	+	+	+	+	*Pediococcus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

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				NaCl con.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:4g	Small shiny, mucoid	C/T	+	+	–	+	+	+	–	+	+	+	+	+	+	*Pediococcus* sp
HFAt:4h	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAt:4i	Small circular, milky white	Rod	+	+	–	+	+	+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4j	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	–	+	+	+	+	*Pediococcus* sp
HFAt:4k	Large, mucoid, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4l	Large, mucoid, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4m	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–.	*Pediococcus* sp
HFAt:4n	Large, mucoid, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4o	Small circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4p	Medium, circular, greyish	Rod	–	+	–	+	+	–	–	+	+	+	+	+	+	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 3 (*Continued*)

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl con.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:4q	Large, mucoid, elevated	Coccus	+	+	–	+	+	+	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4r	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4s	Medium, circular, milky	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4t	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAt:4u	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	–	*Pediococcus* sp
HFAt:4v	Medium, circular, milky	Rod	+	+	–	+	+	+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4w	Small circular, milky white	Rod	–	+	–	+	+	+	–	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4x	Small circular, milky white	Rod	–	+	–	+	+	+	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4y	Large circular, milky white	Rod	–	+	–	+	+	–	+	+	+	+	+	+	–	*Lactobacillus* sp
HFAt:4z	Large circular, milky white	Rod	–	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 3 (*Continued*)

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				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:4A	Large, pale white, elevated	Coccus	+	+	–	+	+	+	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:4B	Large circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4C	Large circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:4D	Medium, circular, greyish	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:5a	Large, pale white, elevated	Ovoid	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5b	Large, pale white, elevated	Coccus	+	+	–	w+	+	w+	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5c	Large, pale white, elevated	Coccus	+	+	–	w+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5d	Large, pale white, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5e	Large, pale white, elevated	Ovoid	+	+	–	+	+	+	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5f	Large, mucoid, elevated	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C ; growth was observed on all the isolates; +, positive results; –, negative results

Table 3 (*Continued*)

Isolates	Colony Morphology	Cell morphology	Arginine hydrolysis	Growth in/at different						Sugar fermentation						Tentative identification
				NaCl con.		Temp.		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:5g	Small circular, pale white	Ovoid	+	+	–	+	+	–	+	+	+	+	+	+	–	*Enterococcus* sp
HFAt:5h	Large, pale white, elevated	Coccus	–	+	–	+	+	+	+	+	+	+	+	+	–	*Enterococcus* sp
HFAt:5i	Large circular, milky white	Rod	+	+	–	w+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:5j	Small circular, mucoid	Coccus	+	+	–	w+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAt:5k	Small shiny, mucoid	C/T	+	+	–	+	+	–	+	+	+	+	+	+	+	*Pediococcus* sp
HFAt:5l	Large circular, milky white	Rod	+	+	–	w+	+	–	–	+	+	+	+	+	–	*Enterococcus* sp
HFAt:5m	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5n	Small circular, pale white	Coccus	+	+	–	w+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5o	Small circular, mucoid	coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Streptococcus* sp
HFAt:5p	Small circular, pale white	Ovoid	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5q	Medium, circular, greyish	Rod	+	+	–	w+	+	–	–	+	+	+	+	+	+	*Lactobacillus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at 15°C; growth was observed at on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

Table 3 (*Continued*)

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				NaCl con.		Temp		pH								
				6.5%	10%	10°C	45°C	3.9	9.6	Galactose	Maltose	Mannitol	Raffinose	Rhamnose	Sucrose	
HFAt:5r	Large circular, milky white	Rod	+	+	–	+	+	–	+	+	+	+	+	+	+	*Lactobacillus* sp
HFAt:5s	Large circular, pale white	Ovoid	+	+	–	w+	+	+	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5t	Small shiny, mucoid	C/T	+	+	–	+	+	+	+	+	+	+	+	+	–	*Pediococcus* sp
HFAt:5u	Small circular, pale white	Coccus	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp
HFAt:5v	Small circular, pale white	Ovoid	+	+	–	+	+	–	+	+	+	+	+	+	+	*Enterococcus* sp

All the isolates were gram positive, catalase negative, non-motile and homofermentative. No growth was observed at 18% NaCl and at growth was observed at 15°C on all the isolates; +, positive results; –, negative results, C/T, Coccus /Tetrad.

5.5. Distribution of LAB

The total of 126 isolates were isolated, among the majority isolates were *Lactobacillus* species (n=52), followed by *Enterococcus* species (n=39), *Pediococcus* species (n=22) and *Streptococcus* (n=13). The distribution of *Lactobacillus* sp belonging to group rare antibiotic user and frequent traditional medicine user were equal (n=26) for each. The count of *Enterococcus* sp and *Pediococcus* sp was higher in the latter group with (n=23) and (n=12) respectively. Whereas in the former group the count of *Enterococcus* sp and *Pediococcus* sp was (n=16) and (n=10) respectively. The number of *Streptococcus* sp identified was higher (n=10) in rare antibiotic user in contrast to frequent traditional medicine user where the count was 3 (figure 1).

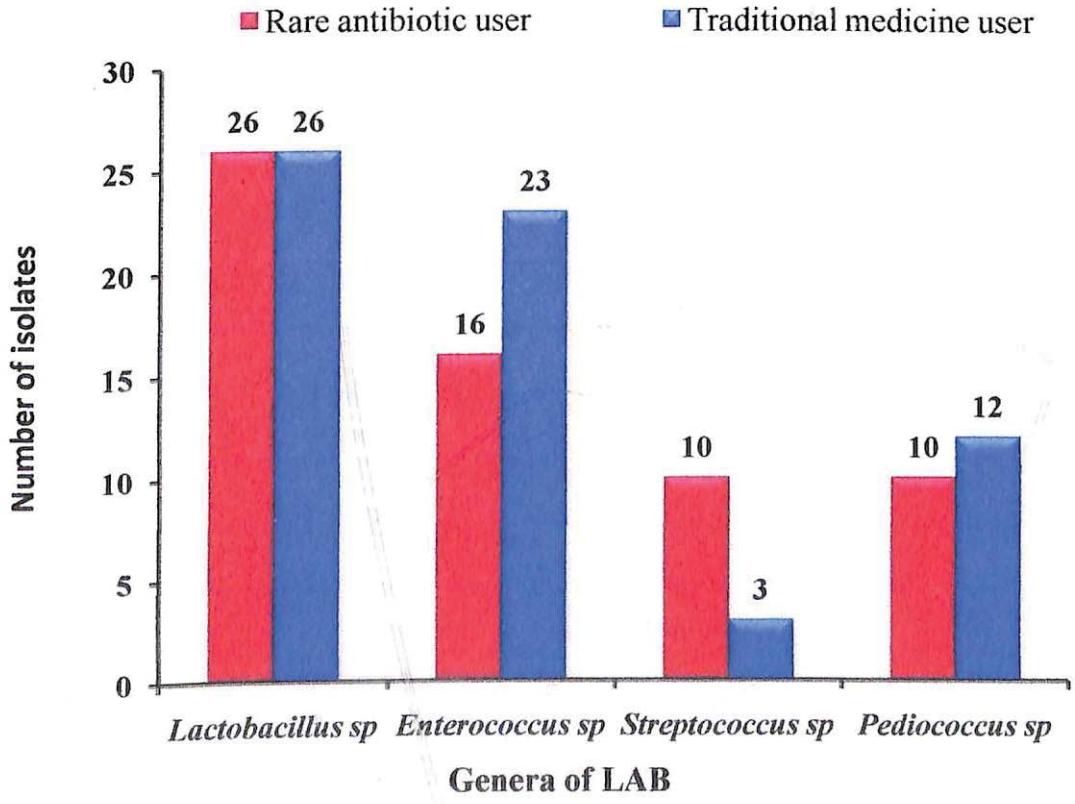

Fig 1: The distribution of Lactic Acid Bacteria in faecal samples of two groups from healthy individuals (i.e. rare antibiotic user and frequent traditional medicine user).

5.6 Results for Antibiotic susceptibility Testing

The strains *Escherichia coli* MTCC 1089, Gram Positive and *Staphylococcus aureus* MTCC 7443 Gram negative strain were taken as control strains. The control was used for every set of tests done. For the test of control strains Muller-Hinton agar was used and the strains were incubated at 37°C for 18- 24 hrs. The zone of inhibition was observed, and the results matched with that recommended by Clinical and Laboratory Standards Institute, CLSI (2010) for reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (**Annexure III**). The results were positive, so it was evident that the tests were properly performed.

Tests were performed with the control strains *E. coli* MTCC 1089 and *S. aureus* MTCC 7443 (Table 4). Results were compared with the standard charts of CLSI (2012). As our results agreed to those of CLSI, 2012 we approved that the method was properly performed.

Table 4: Zone size interpretation of control strains.

Antimicrobial agent	Symbol	Disc Content	*E. coli* MTCC 1089	*S. aureus* MTCC 7443
Amikacin	AK	30 mcg	22mm	25 mm
Ampicillin	AMP	10 mcg	23mm	29 mm
Chloramphenicol	C	30 mcg	27mm	28 mm
Clindamycin	CD	10 mcg	10mm	28 mm
Erythromycin	E	15 mcg	21mm	27mm
Kanamycin	K	5 mcg	15mm	18 mm
Methicillin	MET	5 mcg	0mm	21 mm
Norfloxacin	NX	10 mcg	30mm	26 mm
Penicillin G	P	10 mcg	10 mm	35 mm
Streptomycin	S	10 mcg	0mm	22 mm
Tetracycline	TE	30 mcg	25 mm	28 mm
Vancomycin	VA	30 mcg	<10 mm	18 mm

Note: The zone size were matched with the reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 recommended by CLSI, (2012).

5.6 Results for AST pattern of Lactic acid bacteria.

The isolates which showed variation in the morphological, physiological and biochemical test performed were taken for Antimicrobial Susceptibility Testing (AST). Thus, of 126 isolates, only 73 isolates were tested against 12 antibiotics - ampicillin, amikacin, chloramphenicol, clindamycin, erythromycin, kanamycin, methicillin, norfloxacin, penicillin G, streptomycin, tetracycline and vancomycin,.

5.6.1. AST results for *Lactobacillus* sp.

In *Lactobacillus* sp the observed zone diameter range for susceptible was 21-36mm, for intermediate 16-19 mm and for resistance 0-15 mm (Table 5). The largest zone of inhibition of 36 mm shown by isolates HFAt:2a and HFAt:4w against chloramphenicol and ampicillin, respectively (Table 5). The *Lactobacilli* isolates were not inhibited by kanamycin (aminoglycoside), with no zone of inhibition.

Majority of the isolates identified as *Lactobacillus* sp, from both the categories (rare antibiotic users and frequent traditional medicine users) showed susceptibility towards chloramphenicol, erythromycin, tetracycline, ampicillin, and penicillin G. The *Lactobacillus* isolates from both the categories showed 100% resistance to amikacin, kanamycin, methicillin, and streptomycin.

The percentage of susceptibility to chloramphenicol erythromycin, tetracycline, ampicillin and penicillin G in rare antibiotic user were 100%, 88%, 88% 65% and 82% respectively. In case of clindamycin, 76.47% resistance and 47% susceptibility was observed.

In frequent traditional medicine user susceptibility to chloramphenicol, erythromycin, tetracycline, ampicillin, and penicillin G were 94%, 94%, 59% 76% and 88 % respectively. The observed intermediate zone to ampicillin was 18%, chloramphenicol and erythromycin 6%, clindamycin 29%, tetracycline 35% and vancomycin 23%. The results are tabulated in Table 6 and graphically plotted in fig 2.

Table 5: Zone size in *Lactobacillus* sp using disc diffusion method.

Strain code	Antibiotics										
	AMP	AK	C	CD	E	MET	NX	P	S	TET	VA
Rare antibiotic user											
HFAt:1a	19-I	0-R	24-S	12-R	16-I	0-R	16-I	24-S	0-R	25-S	16-I
HFAt:1f	18-I	0-R	23-S	12-R	17-I	0-R	17-I	22-S	0-R	24-S	16-I
HFAt:1i	21-S	0-R	25-S	12-R	23-S	0-R	15-R	25-S	0-R	23-S	14-R
HFAt:2a	22-S	0-R	36-S	15-R	25-S	0-R	0-R	21-S	0-R	27-S	0-R
HFAt:2b	21-S	12-R	28-S	15-R	22-S	0-R	15-R	25-S	0-R	23-S	14-R
HFAt:2f	26-S	14-R	26-S	16-I	23-S	0-R	0-R	23-S	10-R	25-S	0-R
HFAt:2g	19-I	15-R	27-S	15-R	23-S	0-R	0-R	22-S	12-R	26-S	0-R
HFAr:6a	17-I	13-R	27-S	30-S	26-S	0-R	0-R	18-R	0-R	17-I	0-R
HFAr:6c	26-S	13-R	27-S	10-R	21-S	0-R	0-R	26-S	0-R	28-S	0-R
HFAr:6h	24-S	10-R	24-S	14-R	26-S	0-R	0-R	22-S	0-R	24-S	0-R
HFAr:6i	28-S	0-R	28-S	19-I	27-S	0-R	0-R	22-S	15-R	24-S	0-R
HFAr:6l	17-I	0--R	25-S	15-R	22-S	0-R	0-R	23-S	10-R	21-S	0-R
HFAr:6j	22-S	0-R	26-S	16-R	24-S	0-R	0-R	21-S	0-R	22-S	0-R
HFAr:6m	24-S	0-R	26-S	14-R	23-S	0-R	12-R	22-S	13-R	24-S	0-R
HFAr:6n	21-S	0-R	24-S	16-I	24-S	0-R	0-R	21-S	0-R	24-S	0-R
HFAr:6r	19-I	10-R	23-S	12-R	28-S	0-R	0-R	17-R	0-R	17-I	18-I
HFAr:6J	26-S	0-R	27-S	17-I	27-S	0-R	0-R	22-S	0-R	22-S	0-R
Frequent traditional medicine user											
HFAt:3a	27-S	15-R	28-S	26-S	31-S	12-R	0-R	26-S	14-R	24-S	0-R
HFAt:3c	14-R	11-R	21-S	18-I	23-S	0-R	0-R	23-S	0-R	17-I	0-R
HFAt:3e	21-S	14-R	19-I	18-I	27-S	0-R	0-R	24-S	12-R	19-I	0-R
HFAt:3f	25-S	0-R	27-S	21-S	30-S	0-R	0-R	23-S	13-R	23-S	0-R
HFAt:3g	19-I	12-R	24-S	19-I	30-S	0-R	0-R	17-R	13-R	21-S	0-R
HFAt:3h	18-I	0-R	27-S	16-I	27-S	0-R	0-R	27-S	13-R	21-S	0-R
HFAt:3i	18-I	13-R	30-S	12-R	16-I	0-R	0-R	13-R	12-R	17-I	0-R
HFAt:3l	23-S	15-R	30-S	12-R	26-S	0-R	0-R	23-S	12-R	27-S	0-R
HFAt:4c	29-S	12-R	28-S	24-S	31-S	11-R	0-R	26-S	14-R	25-S	0-R
HFAt:4e	29-S	14-R	29-S	21-S	27-S	<10R	0-R	22-S	13-R	21-S	0-R
HFAt:4p	35-S	14-R	29-S	27-S	35-S	12-R	12-R	31-S	15-R	25-S	0-R
HFAt:4w	36-S	14-R	33-S	28-S	35-S	11-R	0-R	32-S	13-R	22-S	0-R
HFAt:4x	23-S	12-R	27-S	13-R	27-S	0-R	13-R	28-S	13-R	21-S	16-I
HFAr:5i	22-S	10-R	22-S	21-S	23-S	0-R	17-I	23-S	0-R	18-I	19-I
HFAr:5l	25-S	12-R	26-S	24-S	27-S	0-R	0-R	24-S	10-R	0-R	26-S
HFAr:5q	25-S	12-R	28-S	16-I	27-S	0-R	14-I	22-S	0-R	19-I	16-I
HFAr:5r	27-S	0-R	26-S	0-R	24-S	0-R	14-I	22-S	0-R	16-I	17-I

Note: Zone diameter in mm, results were expressed as sensitive, S (21 mm); intermediate, I (16-20 mm) and resistant, R (15 mm), respectively according to that described by Volkova *et al.*, (2006) and Charteris *et al.* (1998).AMP, ampicillin; AK, amikacin; C, chloramphenicol; CD, clindamycin; E, erythromycin; MET, methicillin; NX, norfloxacin; P, penicillin G; S, streptomycin; VA, vancomycin; TET, tetracycline. Results for K, kanamycin not showed (no zone of inhibition).

Table 6: AST pattern of *Lactobacillus* sp from faecal samples in two groups of healthy individuals.

Antibiotics	Symbol	Groups	Susceptible		Intermediate		Resistant	
			No.	%	No.	%	No.	%
Ampicillin	AMP	A	11	65	6	76.	0	0
		T	13	76	3	18	1	6
Chloramphenicol	C	A	17	100	0	0	0	0
		T	16	94	1	6	0	0
Clindamycin	CD	A	0	0	4	24	13	76
		T	8	47	5	29	4	24
Erythromycin	E	A	15	88	2	12	0	0
		T	16	94	1	6	0	0
Norfloxacin	NX	A	0	0	2	12	15	88
		T	0	0	0	0	17	100
Penicillin G	P	A	15	88	0	0	2	12
		T	15	88	0	0	2	12
Tetracycline	TET	A	15	88	2	12	0	0
		T	10	59	6	35	1	6
Vancomycin	VA	A	0	0	3	18	14	82
		T	1	6	4	23	12	71

Note: A stands for "Rare antibiotic user" and T stands for "Frequent traditional medicine user". The results for methicillin, streptomycin, kanamycin and amikacin are not shown (100% resistance) was observed in the isolates from both the groups for these antibiotics.

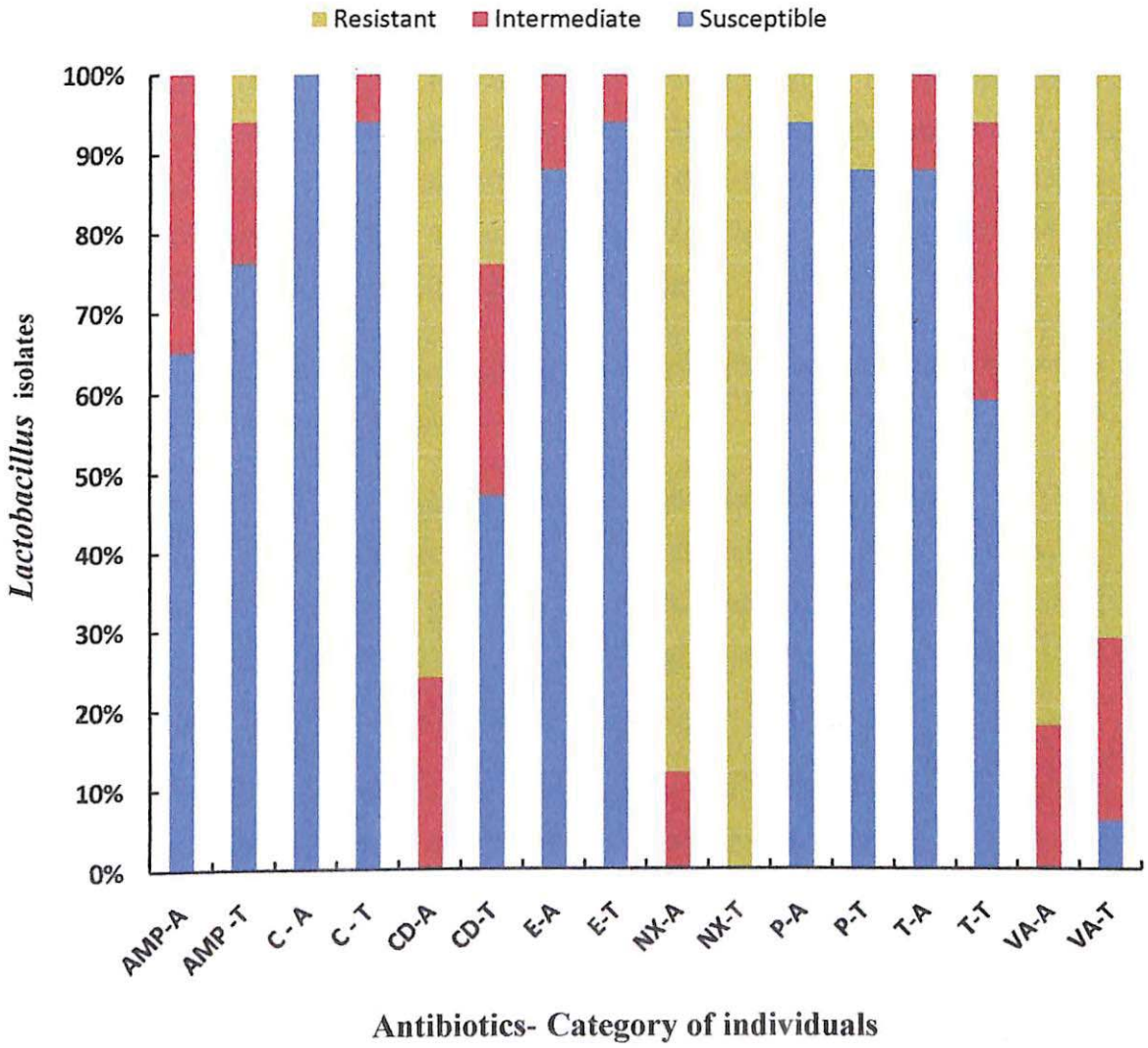

Fig 2: AST pattern of *Lactobacillus* sp from faecal samples in two groups of healthy individuals. A= Rare antibiotic user; T= Frequent traditional medicine user.

5.6.2. AST results for *Enterococcus* sp.

In *Enterococcus* sp the observed zone diameter range for susceptible was 21-31 mm, for intermediate 16-20 mm and for resistance 0-15 mm (Table 7). The largest zone of inhibition of 31mm to chloramphenicol by isolate HFAt:4q was observed and 30 mm zone size was shown by isolate HFAt:1c and HFAr:5n to chloramphenicol and tetracycline, respectively. The isolates of *Enterococcus* sp were not inhibited by kanamycin (aminoglycoside), with no zone of inhibition.

In both the categories, 100% susceptibility to chloramphenicol and 100% resistance against kanamycin and methicillin was observed.

In rare antibiotic user, 100% susceptibility was observed for tetracycline, 57.14 % were susceptible to chloramphenicol followed by ampicillin (85.72%), clindamycin, erythromycin, norfloxacin, and penicillin G, 33.33%. The resistance pattern observed was 66.67% to penicillin G, 33.33% to ampicillin, erythromycin, norfloxacin, and vancomycin and 28.57% to tetracycline.

In frequent traditional medicine user, the percentage of susceptibility was 100% to erythromycin and penicillin G, 85.72% to ampicillin, 57.14% to tetracycline, 14.28% to clindamycin and norfloxacin (14.28%). The results are tabulated in Table 8 and graphically plotted in fig 3.

Table 7: Zone size in *Enterococcus* sp by disc diffusion method.

Strain code	Antibiotics										
	AMP	AK	C	CD	E	MET	NX	P	S	TET	VA
Rare antibiotic user											
HFAt:1b	18-I	0-R	24-S	13-I	23-S	0-R	25-S	0-R	13-R	25-S	0-R
HFAt:1c	25-S	20-I	30-S	0-R	0-R	0-R	17-I	0-R	19-I	24-S	15-I
HFAt:1d	0-R	12-R	32-S	0-R	16-I	0-R	0-R	22-S	13-R	25-S	17-I
Frequent traditional medicine user											
HFAt:3m	21-S	15-R	26-S	0-R	28-S	0-R	0-R	22-S	13-R	22-S	0-R
HFAt:4k	20-I	13-R	25-S	13-R	27-S	12-R	17-I	21-S	<10R	29-S	20-I
HFAt:4q	26-S	12-R	31-S	13-R	26-S	0-R	21-S	27-S	10-R	28-S	10-R
HFAr:5n	21-S	0-R	25-S	16-I	25-S	0-R	0-R	23-S	10-R	30-S	0-R
HFAr:5p	29-S	10-R	25-S	0-R	23-S	13-R	18-I	26-S	10-R	17-I	19-I
HFAr:5s	27-S	15-R	28-S	0-R	24-S	0-R	18-I	28-S	10-R	13-R	18-I
HFAr:5v	23-S	12-R	21-S	14-R	25-S	0-R	17-I	22-S	11-R	0-R	13-R

Note= AMP, ampicillin; AK, amikacin; C, chloramphenicol; CD, clindamycin; E, Erythromycin; MET, methicillin; NX, norfloxacin; P, penicillin G; S, streptomycin; VA, vancomycin; TET, tetracycline. Results were expressed as sensitive, S (≥21 mm); intermediate, I (16-20 mm) and resistant, R (≤15 mm), respectively according to that described by Volkova *et al.* (2006); Charteris *et al.*(1998).

For 'P'zone of inhibition less than 19 mm is R, for 'TET' and 'VA' ≤14 mm; and for 'K' and 'C' ≤13mm is considered R (Charteris *et al.*,1998). Results for "K" not showed (no zone of inhibition).

Table 8: AST pattern of *Enterococcus* sp from faecal samples in two groups of healthy individuals.

Antibiotics	Groups	Susceptible		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Ampicillin	A	1	33.33	1	33.33	1	33.33
	T	6	85.72	1	14.28	0	0
Clindamycin	A	0	0	1	33.33	2	66.67
	T	0	0	1	14.28	6	85.72
Erythromycin	A	1	33.33	1	33.33	1	33.33
	T	7	100	0	0	0	0
Norfloxacin	A	1	33.33	1	33.33	1	33.33
	T	1	14.28	4	57.14	2	28.57
Penicillin G	A	1	33.33	0	0	2	66.67
	T	7	100	0	0	0	0
Tetracycline	A	3	100	0	0	0	0
	T	4	57.14	1	14.28	2	28.57
Vancomycin	A	0	0	2	66.67	1	33.33
	T	0	0	3	42.86	4	57.14

Note: A stands for "Rare antibiotic user" and T stands for "Frequent traditional medicine user". 100% susceptible to chloramphenicol and 100% resistance to kanamycin and methicillin in both groups (results not shown).

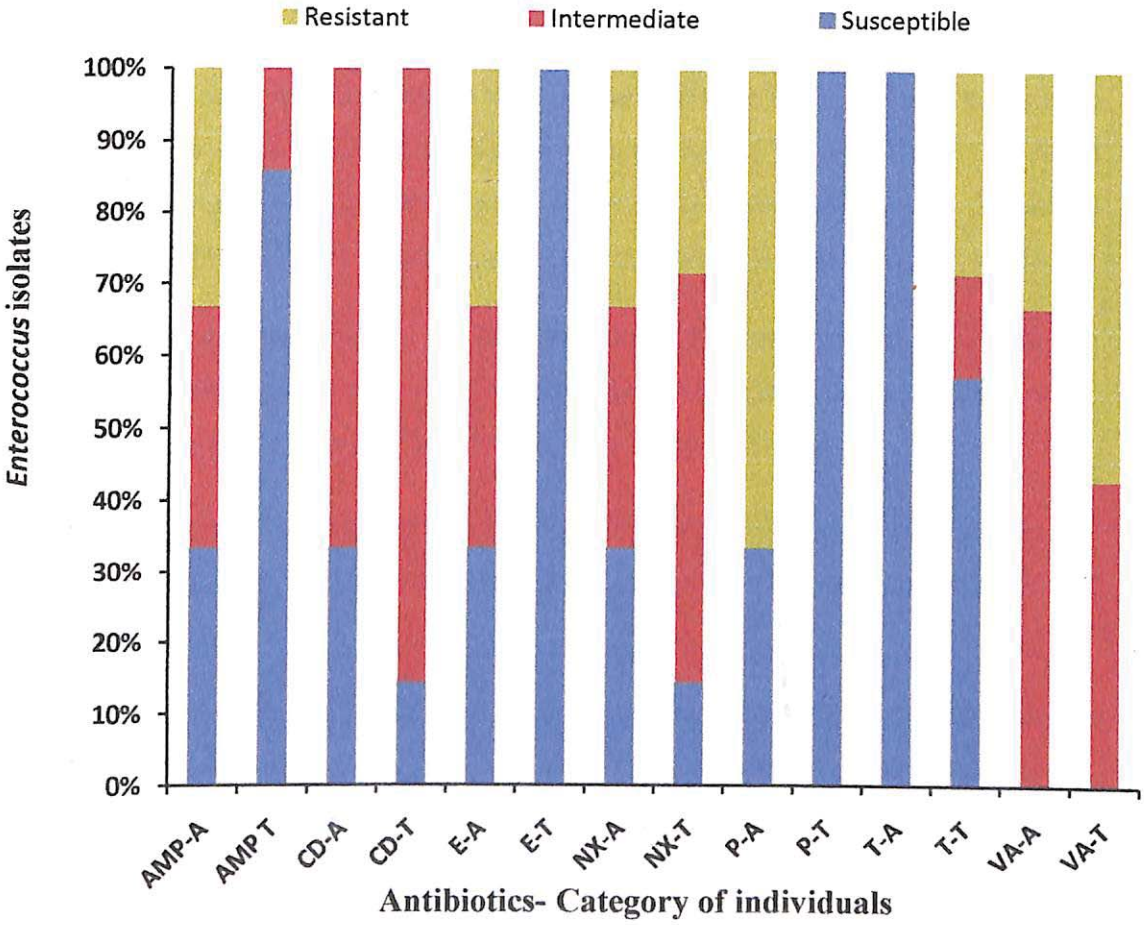

Fig 3: AST pattern of *Enterococcus* sp from faecal samples in two groups of healthy individuals. A= Rare antibiotic user; T= Frequent traditional medicine user.

5.6.3. AST results for *Streptococcus* sp.

The zone diameter observed in *Streptococcus* sp ranged from 21-18mm. the largest zone of inhibition observed 28mm to tetracycline by isolate HFAt:2j followed by zone size 26mm for tetracycline and erythromycin by isolate HFAt:6k and HFAt:6d respectively from rare antibiotic user. In another group zone size 26mm to chloramphenicol and 25mm to tetracycline was observe in isolate HFAṛ:5o, Table 9.

In both the categories 100% of the *Streptococcus* isolates were susceptible to chloramphenicol, penicillin G and tetracycline and 100% of the isolates were resistance to amikacin, kanamycin, methicillin, and streptomycin.

The isolates from rare antibiotic user showed 50% susceptibility to erythromycin and 66.67% to ampicillin. The resistance to clindamycin and norfloxacin was 66.67% and 50% to vancomycin.

The isolates from frequent traditional medicine user showed 100% susceptibility to erythromycin and ampicillin and resistance to vancomycin and clindamycin is also 100%. Intermediate zone of 100% was observed to norfloxacin. The results are tabulated in Table 10 and graphically plotted in fig 4.

Table 9. Zone size in *Streptococcus* sp. by disc diffusion method.

Isolates	Antibiotics									
	AMP	AK	C	CD	E	NX	P	S	TET	VA
Rare antibiotic user										
HFAt:1g	19-I	0-R	24-S	12-R	16-I	17-I	24-S	0-R	24-S	15-I
HFAt:1h	18-I	0-R	25-S	13-R	15-R	15-R	23-S	0-R	24-S	16-I
HFAt:2h	21-S	12-R	24-S	14-R	22-S	0-R	22-S	0-R	24-S	0-R
HFAt:2j	21-S	14-R	24-S	14-R	23-S	20-I	23-S	10-R	28-S	0-R
HFAr:6d	24-S	13-R	24-S	16-I	26-S	0-R	23-S	0-R	24-S	17-I
HFAr:6k	20-I	0-R	26-S	16-I	23-S	0-R	22-S	0-R	26-S	0-R
Frequent traditional medicine user										
HFAr:5o	23-S	0-R	26-S	12-R	23-S	17-I	25-S	11-R	25-S	0-R

Note= AMP, ampicillin; AK, amikacin; C, chloramphenicol; CD, clindamycin; E, Erythromycin; MET, methicillin; NX, norfloxacin; P, penicillin G; S, streptomycin; VA, vancomycin; TET, tetracycline. Results were expressed as sensitive, S (≥21 mm); intermediate, I (16-20 mm) and resistant, R (≤15 mm), respectively according to that described by Volkova *et al.* (2006); Charteris *et al.* (1998). For 'P' zone of inhibition less than 19mm is Resistant, for 'T' and 'V' ≤14 mm and for Kanamycin 'K' and 'C' ≤13mm is considered resistant (Charteris *et al.*,1998a). Results for 'K' not showed (no zone of inhibition).

Table 10: AST pattern of *Streptococcus* sp from faecal samples in two groups of healthy individuals.

Antibiotics	Symbol	Groups	Susceptible		Intermediate		Resistant	
			No.	%	No.	%	No.	%
Ampicillin	AMP	A	3	50	3	50	0	0
		T	1	100	0	0	0	0
Clindamycin	CD	A	0	0	2	33.33	4	66.67
		T	0	0	0	0	1	100
Erythromycin	E	A	4	66.67	1	16.66	1	16.66
		T	1	100	0	0	0	0
Norfloxacin	NX	A	0	0	2	33.33	4	66.67
		T	0	0	1	100	0	0
Vancomycin	VA	A	0	0	3	50	3	50
		T	0	0	0	0	1	100

Note: A stands for "Rare antibiotic user" and T stands for "Frequent traditional medicine user". The results for AK, K, MET and S not shown as 100% resistance was observed and results for C, P and TET not shown as 100% susceptibility was observed in both the groups.

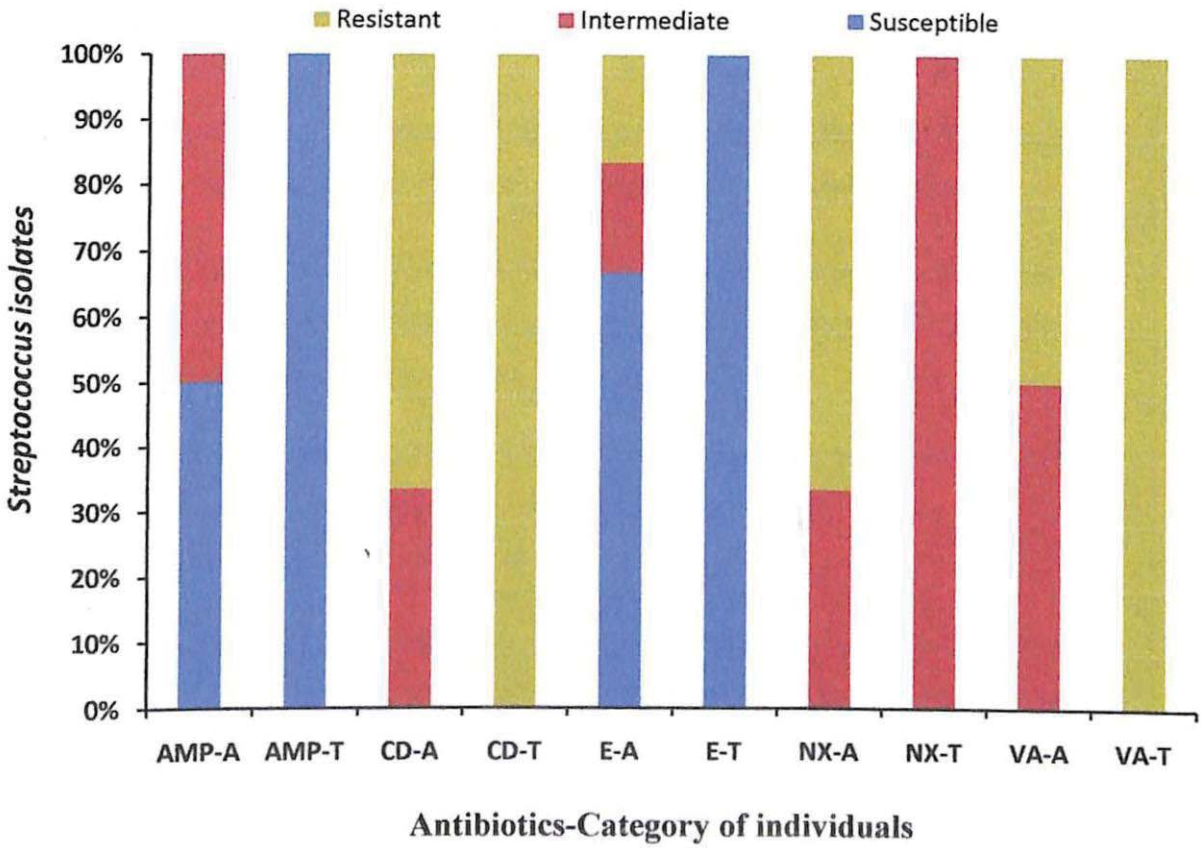

Fig 4: AST pattern of *Streptococcus* sp from faecal samples in two groups of healthy individuals. A= Rare antibiotic user; T= Frequent traditional medicine user

5.6.4. AST results for *Pediococcus* sp

The largest zone diameter observed in *Pediococcus* sp from frequent traditional medicine user was 35 mm and 34 mm to ampicillin and chloramphenicol respectively by isolate HFAt:4m., followed by zone diameter of 32mm to tetracycline by isolate HFAt:3b and HFAt:4b, Table 11.

In rare antibiotic user the largest zone diameter observed was 32mm to tetracycline by isolate HFAr:6N followed by 30mm to chloramphenicol by isolate HFAr:6p.

The susceptibility of 100% was observed to chloramphenicol, penicillin G and tetracycline and 100% resistance against amikacin, kanamycin, methicillin and streptomycin in isolates of both groups.

In rare antibiotic user 88.88% and 91.67% susceptibility was observed to ampicillin and erythromycin, respectively. The resistance pattern was 77.78% to clindamycin, 58.33% to norfloxacin and 41.67%% to vancomycin.

The isolates from frequent traditional medicine user showed higher susceptibility to ampicillin 91.67%, erythromycin 100%. The resistance pattern to clindamycin 83.33%, 66.67% to norfloxacin and 44.44% to vancomycin. The results are tabulated in Table 12 and graphically plotted in fig 5.

Table 11: Zone size in *Pediococcus sp* by disc diffusion method.

Isolates	Antimicrobial agent										
	AMP	AK	C	CD	E	MET	NX	P	S	TET	VA
Rare antibiotic user											
HFAr:6p	16-I	12-R	30-S	13-R	26-S	10-R	15-R	23-S	13-R	28-S	14-R
HFAr:6s	23-S	13-R	24-S	20-I	26-S	0-R	20-I	21-S	11-R	28-S	19-I
HFAr:6A	27-S	0-R	24-S	18-I	26-S	0-R	15-R	24-S	11-R	29-S	0-R
HFAr:6C	23-S	0-R	26-S	0-R	27-S	0-R	16-I	2I-S	0-R	28-S	15-I
HFAr:6E	23-S	0-R	25-S	0-R	25-S	0-R	0-R	22-S	<10R	29-S	14-R
HFAr:6H	22-S	0-R	25-S	0-R	27-S	0-R	0-R	21-S	0-R	25-S	0-R
HFAr:6L	22-S	0-R	25-S	0-R	26-S	0-R	15-R	21-S	0-R	28-S	16-I
HFAr:6M	24-S	0-R	26-S	0-R	26-S	0-R	15-R	21-S	0-R	29-S	15-I
HFAr:6N	22-S	12-R	24-S	13-R	28-S	0-R	18-I	21-S	>10R	32-S	20-I
Frequent traditional medicine user											
HFAt:3b	22-S	10-R	27-S	14-R	31-S	12-R	0-R	21-S	14-R	32-S	20-I
HFAt:3o	26-S	0-R	26-S	0-R	18-I	18-I	0-R	29-S	0-R	30-S	16-I
HFAt:4b	21-S	12-R	23-S	14-R	28-S	0-R	17-I	32-S	10-R	32-S	18-I
HFAt:4f	17-I	0-R	25-S	13-R	23-S	0-R	18-I	26-S	13-R	29-S	0-R
HFAt:4g	26-S	12-R	26-S	11-R	24-S	0-R	15-R	25-S	10-R	26-S	0-R
HFAt:4h	30-S	13-R	31-S	13-R	26-S	11-R	14-R	28-S	14-R	28-S	0-R
HFAt:4m	35-S	14-R	34-S	27-S	35-S	12-R	0-R	31-S	15-R	31-S	0-R
HFAt:4j	22-S	10-R	27-S	13-R	26-S	0-R	20-I	21-S	10-R	21-S	19-I
HFAt:4t	25-S	10-R	26-S	13-R	27-S	<10R	19-I	21-S	<10R	30-S	20-I
HFAt:4u	25-S	10-R	26-S	12-R	26-S	0-R	18-I	21-S	10-R	28-S	19-I
HFAr:5k	21-S	10-R	24-S	17-I	25-S	0-R	11-R	21-S	<10R	29-S	14-R
HFAr:5t	22-S	0-R	25-S	12-R	23-S	0-R	0-R	22-S	<10R	28-S	17-I

Note: The results for kanamycin not shown, as no zone of inhibition was observed in all the isolates. Results were expressed as sensitive, S (≥21 mm); intermediate, I (16-20 mm) and resistant,R (≤15 mm), respectively according to that described by Volkova *et al.*, (2006); Charteris *et al.*, (1998a). For penicillin G zone of inhibition less than 19mm is Resistant, for tetracycline and vancomycin ≤14 mm and for kanamycin and chloramphenicol ≤13mm is considered resistant (Charteris *et al.*, 1998).AMP= Ampicillin, Ak= Amikacin, C= Chloramphenicol, C=clindamycin, E= Erythromycin, MET= methicillin, Nx= Norfloxacin, P= Penicillin G, S= Streptomycin, TET= Tetracycline, VA= Vancomycin.). Results for 'K' not showed (no zone of inhibition).

Table 10: AST pattern of *Pediococcus* sp from faecal samples in two groups of healthy individuals.

Antibiotics	Symbol	Groups	Susceptible		Intermediate		Resistant	
			No.	%	No.	%	No.	%
Ampicillin	AMP	A	8	88.89	1	11.11	0	0
		T	11	91.67	1	8.33	0	0
Clindamycin	CD	A	0	0	2	22.22	7	77.78
		T	1	8.33	1	8.33	10	83.33
Erythromycin	E	A	9	100	0	0	0	0
		T	11	91.67	1	8.33	0	0
Norfloxacin	NX	A	0	0	3	33.33	6	66.67
		T	0	0	5	41.67	7	58.33
Vancomycin	VA	A	0	0	5	41.67	4	44.44
		T	0	0	7	58.33	5	41.67

Note: A stands for "Rare antibiotic user" and T stands for "Frequent traditional medicine user". The results for AK, K, MET and S not shown as 100% resistance was observed and results for C, P and TET not shown as 100% susceptibility was observed in both the groups.

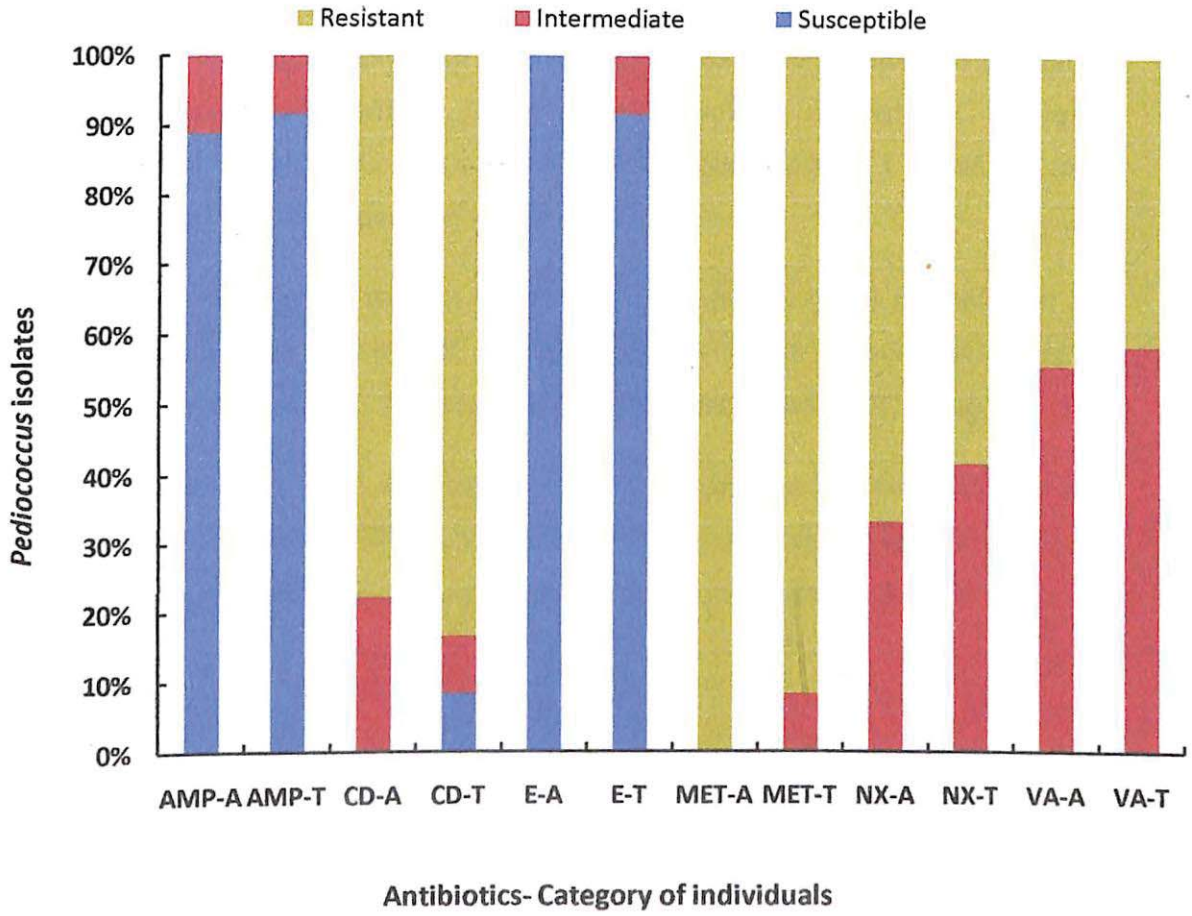

Fig 5: AST pattern of *Pediococcus* sp from faecal samples in two groups of healthy individuals. A= Rare antibiotic user; T= Frequent traditional medicine user.

5.7 MIC results for *Lactobacillus* sp

The *Lactobacillus* isolates was subjected to antimicrobial agent vancomycin and streptomycin and chloramphenicol for MIC. The MIC of 25 isolates was performed, (n=15) isolates, showed MIC > 16 µg/ml, (n=3) isolates, showed MIC 16 µg/ml, (n=4) isolates showed MIC 4 µg/ml, (n=2) isolates showed MIC 1 µg/ml and (n=1) isolate showed MIC 0.5 µg/ml for vancomycin.

For streptomycin, (n=10) isolates showed MIC 512 µg/ml, (n=5) showed MIC 256 µg/ml, (n=4) isolates showed MIC 128 µg/ml, (n=3) isolates showed MIC 64 µg/ml, (n=2) isolates showed MIC 32 µg/ml and (n=1) isolates showed MIC 16 µg/ml.

For chloramphenicol, (n=6) isolates showed MIC 2µg/ml, (n=3) isolates showed MIC 1 µg/ml, (n=10) showed MIC 0.5 µg/ml, (n=2) isolates showed MIC 4 µg/ml and (n=4) isolates showed MIC <0.5 µg/ml. The result is shown below in table 13.

Table 13: Minimum Inhibitory Concentration of selected antimicrobial substances for *Lactobacillus* sp.

Sl. No	Isolate Code	Antimicrobial agents (µg/ml)		
		Vancomycin	Streptomycin	Chloramphenicol
1	HFAr:5r	>16	32	1
2	HFAr:5i	>16	32	0.5
3	HFAr:6i	>16	256	0.5
4	HFAt:3c	>16	512	2
5	HFAt:4c	16	512	1
6	HFAr:6j	>16	512	0.5
7	HFAt:3a	0.5	128	0.5
8	HFAr:5l	>16	256	1
9	HFAt:2f	4	64	2
10	HFAt:2a	16	512	4
11	HFAt:3l	4	16	2
12	HFAt:6h	>16	128	<0.5
13	HFAr:6c	1	256	0.5
14	HFAr:5q	4	64	0.5
15	HFAr:6h	16	512	0.5
16	HFAt:4p	4	128	<0.5
17	HFAr:6m	>16	256	<0.5
18	HFAt:4e	>16	256	0.5
19	HFAt:2b	1	512	2
20	HFAr:6n	>16	512	4
21	HFAr:6r	>16	512	2
22	HFAr:6j	>16	64	0.5
23	HFAt:3g	>16	128	0.5
24	HFAt:3e	>16	512	<0.5
25	HFAt:4w	>16	512	2

Note: Range of antibiotics- vancomycin (1-16µg/ml), streptomycin (0.25-4096µg/ml), chloramphenicol (0.25-256 µg/ml). The microbial breakpoints are 2, 16 and 4 respectively according to (European Commission. 2008).

Chapter - VI

Discussion

6. Discussion

In a place like Sikkim, people of diverse ethnicity, culture and food habits are found. The present study was conducted on the people of Arithang and Aritar (East district) of Sikkim. The main objective of this study was to isolate lactic acid bacteria from healthy individuals, and perform Antibiotic susceptibility of those isolates. From 126 isolates, 62 isolates were isolated from rare antibiotic user, and 64 isolates from traditional medicine user.

Most of the Sikkim's populations are non-vegetarian with only 11.7% reporting as vegetarian (Tamang *et al.*, 2007). The fermented foods are a significant part of the diet of people of Sikkim and the per capita consumption of fermented food and beverages is 163.8 g/day, resulting 12.6% of total food intake (Tamang *et al.*, 2007).

As per the survey done through questionnaire, in this study two individuals consumed fermented alcoholic beverages on regular basis. Almost all the individuals were found to consume fermented foods like Gundruk, Sinki, Kinema and Curd. In total 25 individuals were surveyed but we could include only few individuals in our study as only these many individuals met the criteria of healthy individuals. These individuals had no record of frequent medication, past history of severe infection, they were never hospitalised and had a healthy life style. As, LAB plays a crucial role in maintaining the ecological balance in GI tract, factors such as food habits , antimicrobial agents, stress can may affect the normal GI flora.

6.1. Microbial analysis

The isolation of LAB was done from stool samples of healthy individuals, the count of LAB in stool ranged from 9.66±0.57 log CFU/g to 10.33±0.57 log CFU/g. The total of 6 have samples was included in our study and isolated 126 isolates from these samples. In the present study, the dominant LAB isolated from stool sample was *Lactobacillus* sp 52 (41.27%), followed by *Enterococcus* sp 39 (30.95%), *Pediococcus* sp 22 (17.46%) and *Streptococcus* sp 13 (10.31%). According to Anderson *et al.*, (2006) to effectively sample the bacterial populations, it is necessary to isolate large number of isolates per individuals. It has been studied that for comparing the enterococcal populations from animals, humans and environmental samples, taking few samples but analyzing maximum isolates per sample could give

better results than taking more samples and few isolates per sample (Kuhn *et al.*, 2003).

The distribution of LAB in rare antibiotic user and traditional medicine user were compared. The *lactobacillus* sp distribution was similar in the both groups. But the distribution of *Enterococcus* and *Pediococcus* was found be higher in traditional medicine user with the number of isolates 23 and 12, in rare antibiotic user the number was 16 and 10, respectively. In contrast, the population of *Streptococcus* sp was 10 in rare antibiotic user and 3 in traditional medicine user. The present study contrast with the similar study by Kilic, (2009) in which out of 107 LAB, 36 isolates of *lactobacillus* sp and 68 isolates *Enterococcus* sp were identified.

Enterococcus sp are found to be most predominant genera in infants (Kirjavainen *et al.*, 2000) but also prevalent in adults. Among large number of commensal bacteria present in GIT of mammals, *Enterococci* are also common commensal of healthy human and animals (Vakerckhoven *et al.*, 2004).

In different studies it was found that in healthy samples the average predominant *Enterococci* are *E. faecalis* 54.9% and *E. faecium.* 43.4% (Guimaraes *et al.*, 2009; Novais *et al.*, 2003; Poeta *et al.*, 2005). These two species of *Enterococcus* are also commonly used as probiotics (Gardnier *et al.*, 2002). In our study isolates were identified to genus level as sugar fermentation test was done for only 6 sugars. Nevertheless we assume that the majority of the isolates were *E. faecalis* as the samples were taken from healthy individuals. The limitation in our study lies as a single medium MRS was used as such we could determine the quantitative analysis of only some local bacterium.

6.2. Zone of inhibition

The antibiotic susceptibility test was performed for 12 antibiotics: amikacin, streptomycin, kanamycin (aminoglycosides), chloramphenicol (phenicols) clindamycin (lincosamide), erythromycin (macrolides), ampicillin, methicillin, penicillin G (B- lactams), vancomycin (glycopeptides), tetracycline (tetracyclines) and norfloxacin using disc diffusion method for 73 isolates. The general overview of antibiotic susceptibility pattern by disc diffusion in LAB isolates showed higher susceptibility to ampicillin, penicillin G, chloramphenicol, tetracycline, and erythromycin. In contrast, the antibiotics such as amikacin, kanamycin, streptomycin, methicillin, norfloxacin, vancomycin and clindamycin showed high resistance.

Clindamycin, vancomycin and norfloxacin also showed moderate inhibitory effects on many isolates.

In the similar work done by Majhenic and Matizasicin 2001 using disk diffusion method, *Lactobacilli* were most sensitive to erythromycin, ampicillin and penicillin, where zones ranged from 22-37 mm. In present study, *Lactobacilli* showed almost similar sensitivity for these antimicrobial agents with zone ranging from 21- 35mm, while one of the *Lactobacilli* isolate was resistant to erythromycin. It has been reported that *Lactobacillus* sp are generally susceptible to protein synthesis inhibitors such as chloramphenicol (Coppola *et al.*, 2005; Klare *et al.*, 2007) and to many cell wall synthesis inhibitors such as penicillins and ampicillin (Danielsen and Wind, 2003; Coppola *et al.*, 2005).

In our study one isolate of *Lactobacillus* sp (HFAt:3e), was found to have intermediate susceptibility with zone diameter, 19mm, rest of the isolates were susceptible to Chloramphenicol. For *Pediococcus*, *Enterococcus* and *Streptococcus* the sensitivity were within the zone size, 21-32mm, 21-30mm, 21-26mm respectively

Pediococcus showed some zone of inhibition ranging from (16-20mm), showing intermediate patterns in most *Pediococcus* sp. Pediococcus sp showed high resistance to kanamycin, streptomycin, methicillin and amikacin, HFAt:4m was susceptible to clindamycin.

6.3. AST pattern in LAB

All the isolates in the present study which belongs to the genus *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Streptococcus* showed highest percentage of susceptibility towards chloramphenicol and highest percentage of resistance towards methicillin, streptomycin, kanamycin and amikacin, in comparison to the 12 antibiotics tested.

The resistance to chloramphenicol was not observed in any of the isolates both in disc diffusion method and MIC by agar dilution method in contrast to similar study done by Katla *et al.* (2001) and Ammor *et al.* (2008b) where 3.3% resistance was observed to chloramphenicol. The resistance to kanamycin, norfloxacin and streptomycin seems to be as a rule (Danielsen and Wind, 2003). The possible reason for such

resistance could be the complex intrinsic character that includes cell wall structure and composition or metabolic properties (Kastner *et al.*, 2006).

In our study too, resistance is observed against above mentioned antibiotics kanamycin, norfloxacin, streptomycin and also to clindamycin, methicillin and vancomycin. Nevertheless the human and animal intestinal flora could carry a reservoir for these antibiotic resistance and transferrable genes, and when contaminations with faecal bacteria and the genes occur, there lies a great risk of transmission of these genes (Kastner *et al* ., 2006). Currently, most focus is imparted to clinically relevant bacteria, now it is stated that commensal bacteria largely significant, as it can transfer resistant genes to pathogenic microorganisms (Mathur and Singh, 2005; Ruzauskas *et al.*, 2009). Considering LAB the only major relevant concern is the transferability of antimicrobial resistance (Zdolec *et al.*, 2011).

However, there are indications that the medium MRS may exhibit antagonistic effects with supplemental antimicrobials in susceptibility testing (Huys *et al.*, 2002). Klare *et al.*, (2005) developed lactic acid bacterium susceptibility test medium (LSM), a mixed formulation of IST broth (90% v/v) and MRS broth (10% v/v), we have used the media with some modifications Hi-sensitivity test agar (90%w/v) and MRS agar (10% w/v).

6.3.1. *Lactobacilli*

In the present study, 100% resistance was observed in *Lactobacilli* in both the categories of samples (rare antibiotic user and traditional medicine user) for methicillin (beta- lactams), streptomycin, kanamycin and amikacin (aminoglycosides) which could be due to their natural resistance potential to these antibiotics. Although less, but resistance was also observed in antibiotics such as tetracycline 6%, penicillin G 12%, and ampicillin 6%, in traditional medicine user which generally shows susceptibility to LAB. The resistance could be due to the ingestion of food with bacteria that possess resistance bacteria (Jacobsen *et al.*, 2007), there are reports of food acting a as a source of transmission of bacterium with antibiotic resistance genes. It was observed that high percentage of *Lactobacilli* from rare antibiotic user was susceptible to chloramphenicol 100%, followed by erythromycin and tetracycline 88%, penicillin G 82% and ampicillin 65%. It is reported that *Lactobacillus* sp are generally susceptible to chloramphenicol, clindamycin and erythromycin (Coppola

et.al., 2005, Klare *et al.*, 2007) , in case of clindamycin our study shows contrast as 76% and 24% resistance is observed in rare antibiotic user and traditional medicine user respectively. In *Lactobacilli* from traditional medicine user, high percentage of susceptibility was observed in chloramphenicol and erythromycin (94%), followed by penicillin G 82%, ampicillin 76%, tetracycline 59% and vancomycin 6%. Tetracycline which generally is shown to inhibit LAB shows 6% resistance, which could be due to *Tet* transposable elements that are highly mobilisable (Clewell *et al.*, 1995; Rice, 1998). The similar association is also reported for erythromycin resistance, in LAB mainly associated with food (Mathur and Singh, 2005; Ammor *et al.*, 2007)

The resistant pattern to methicillin, streptomycin, kanamycin and amikacin was 100% in both the groups. In rare antibiotic user the resistance pattern to other antibiotics such as norfloxacin was 88%, vancomycin 82%, clindamycin 76% and ampicillin 6%. In other group the resistant to norfloxacin was highest 100% followed by vancomycin 71%, clindamycin 24%, tetracycline and ampicillin 6%. The resistance pattern of kanamycin by *Lactobacilli* shows similar results with the previous work done (Charteris *et al.*, 1998, Canzek *et al.*, 2001). Majority of *Lactobacilli* also showed resistance to vancomycin a glycopeptides, similar result was reported in a study done by Korhonen *et al.*, 2010). According to their study, *Lactobacilli* other than obligate heterofermenters are generally resistant to vancomycin. It has also been stated that resistance to vancomycin are intrinsic (Tynkkynen *et al.*, 1998), due to the presence of d-Ala-d-lactate in their peptidoglycan instead of the normal dipeptide d-Ala-d-Ala (Ammor *et al.*, 2008a).

As per study done by Korhonen *et al.* (2008), in *Lactobacillus* sp tetracycline resistance are quite frequent (Korhonen *et al.*, 2008), in the present study only one isolate of *lactobacillus* species (HFAr:5l) was resistant to tetracycline from rare antibiotic user. In order to confirm resistance particularly with tetracycline, molecular methods are suggested to know the resistance mechanism (Korhonen *et al.*, 2010). In our study, among *Lactobacilli* species resistance to kanamycin and streptomycin was observed which justify the study done by various researchers where resistant to an aminoglycosides such as kanamycin, streptomycin and gentamycin are frequently observed in *Lactobacilli* (Katla *et al.*, 2001; Danielsen and Wind, 2003: Coppola *et al.*, 2005; Zhou *et al.*, 2005). *Lactobacilli* group is known to present antimicrobial

resistance to some antibiotics, in particular, chloramphenicol (Lin *et al.*, 1996), erythromycin (Cataloluk and Gogebakan, 2004) and tetracycline (Kastner *et al.*, 2006) which are partially found in our study.

6.3.2. *Enterococcci*

In *Enterococcus* sp high susceptibility is shown by ampicillin, chloramphenicol and erythromycin in both the groups, with less susceptibility to penicillin G in rare antibiotic user 33.33%. Resistance strains of *Enterococcus* sp to clindamycin was reported in an experiment done by Korhonen *et al.* 2010. In our study, 76% and 24% resistance is observed in rare antibiotic user and traditional medicine user respectively. In present study all the isolates of *Enterococcus* was completely resistant to methicillin and kanamycin. Resistance to vancomycin found in *Enterococci* could be transmissible, which are plasmid mediated (Leclercq *et al.*, 1992).

Although isolates of LAB showed resistance to many antibiotics, their higher susceptibility to erythromycin and Penicillin G, indicates that the other isolates of LAB are not potential reservoir for transmissible genes towards penicillin G and erythromycin (Korhonen *et al* ., 2010).

6.3.3. Pediococci and *Streptococci*

In present study *Pediococcus* sp showed 100% susceptibility to chloramphenicol, Penicillin G and tetracycline in both the groups for erythromycin the susceptibility was 100% in rare antibiotic user whereas 91.67% in frequent traditional medicine user. In a contrast to the study done by Temmerman *et al.* 2002 in food isolates, where resistance towards tetracycline (38%), penicillin (25%) and chloramphenicol (38%) was reported. Although in this study 8.33% intermediate susceptibility was observed for erythromycin. Their study also reported 100% resistance to vancomycin, which also slightly shows contrast with our study where 44% and 41.67% resistance in rare antibiotic user and traditional medicine user, respectively was observed. We have discussed the results from food isolates presented by precious authors as sufficient papers are not published on AST from stool isolates for *Pediococcus* sp.

In present study, 77.78% and 83.33% resistance to clindamycin was observed in latter and former group respectively. The genus *Pediococcus* showed 100% susceptibility to

chloramphenicol, Penicillin G and tetracycline in both the groups. Resistance strains in *Pediococcus* sp to clindamycin was also reported in an experiment done by Korhonen *et al.* (2010).

The susceptibility to chloramphenicol, penicillin G and tetracycline and resistance to amikacin, ampicillin, methicillin and streptomycin was 100% in both the groups. Towards erythromycin, 16.66% resistance was observed in rare antibiotic user.

6.4. Minimum Inhibitory Concentration

MIC for 25 isolates was performed against antibiotics vancomycin (1-16µg/ml), streptomycin (0.25-4096µg/ml), chloramphenicol (0.25-256µg/ml) following agar dilution method. Although the minimum inhibitory concentrations (MIC) are defined for clinically important microorganisms, internationally valid MICs for *Lactobacilli* have not been determined yet (Duskova and Renata, 2013).

The MIC range of *Lactobacillus* sp for antibiotics chloramphenicol, streptomycin and vancomycin ranged from <0.5-4µg/ml, 32-512µg/ml and <0.5->16µg/ml, respectively. In a study done by Rojo-Bazares *et al.* (2006), the MIC range for chloramphenicol ranged from (≤0.5- 16µg/ml) for LAB strains, in present study the range of MIC for chloramphenicol ranged from <0.5 to 4µg/ml, our results show less MIC. In a similar study done by Klare *et al.* 2007, all the LAB isolates were inhibited by ≤4µg/ml chloramphenicol. But the method they followed was broth microdilution whereas; in this study agar dilution method was used.

The aminoglycoside streptomycin in our study showed higher MIC range from 16-512 µg/ml, which shows similar results with the previous work of Rojo-Bazares *et al.* (2006) and Elkins and Mullis (2004). In total 10 isolates showed high level of resistance with MIC of 512µg/ml. In the study done by Klare *et al.*, 2007 high level of resistance was observed on three probiotic strains of *Lactobacillus* with MIC of 2048 mg/ml.

The glycopeptide vancomycin showed MIC range from 0.5 to >16µg/ml. In different studies the MIC range for vancomycin showed variation, in an experiment performed by Klare *et al.* 2007, for *Lactobacillus* sp the MIC range was >1284µg/ml. The resistance by *Lactobacillus* sp to vancomycin is regarded as intrinsic. Therefore, it could be said that LAB own natural non transmissible resistance, significantly to

vancomycin (Bernardeau *et al.*, 2008). The limitation in our study is that the MIC range performed for vancomycin was limited to 0.5 to >16µg/ml.

According to Elkins and Mullis (2004) *Lactobacilli* show resistance to aminoglycosides because of the membrane impermeability. The resistance to glycopeptides, aminoglycosides have been well documented in the LAB strains in previous studies (Elkins and Mullis, 2004; Mathur and Singh, 2005), they all have complemented that natural and intrinsic resistance was the factor involved, the reason explained is the cell wall structure, including membrane impermeability and sometimes efflux mechanism was also involved (Elkins and Mullis, 2004).

Chapter - VII

Summary

7. Summary

The LAB were isolated from GIT of healthy individuals from stool samples. The samples were collected from the individuals that belonged to age group 25-35 and 35-45. Firstly, the field survey was done based on the designed questionnaire (**Annexure I**) to know the health status of the participants. After the survey, sample was collected and processed to isolate the LAB from the faecal samples. According to the survey done, the isolates were grouped into two categories: from 1) Rare antibiotic user and 2) frequent traditional medicine user. Accordingly, the phenotypic identification was done. The majority of the isolates identified belonged to genera *Lactobacillus*, followed by *Enterococcus*, *Pediococcus* and *Streptococcus*.

The selected isolates based on the difference on their physiochemical properties were subjected to antibiotic susceptibility testing against 12 antibiotics by disc diffusion method. Majority of the isolates from both the groups were highly susceptible to chloramphenicol, followed by tetracycline, penicillin G, erythromycin and ampicillin. Resistance towards kanamycin was 100%, majority also showed high resistance to methicillin, streptomycin, clindamycin, norfloxacin, amikacin and vancomycin, with some variation in the resistance pattern.

As majority of the isolates were *Lactobacilli*, 25 *Lactobacilli* isolates were subjected to MIC to antibiotics vancomycin, streptomycin and chloramphenicol. The maximum MIC range for streptomycin was 512 µg/ml for vancomycin >16 µg/ml and for chloramphenicol 4 µg/ml.

Chapter - VIII

Conclusion

8. Conclusion

The present study focuses on the isolation of LAB from GIT of healthy individuals. The two groups of population were examined: rare antibiotic or allopathic users and traditional medicine users. From the faecal samples 126 isolates were isolated. Identification of these isolates were done on the basis of morphological, physiological, phenotypical and biochemical characteristics in accordance to the taxonomic keys of Bergey's Manual (Sneath *et al.*, 1986), The Prokaryotes (Dworkin *et al.*, 2006); (Simpson and Taguchi, 1995); (Wood and Holzapfel, 1995). The genus of the isolates was identified as *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Enterococcus*. The maximum number of isolates belonged to *Lactobacillus* sp, followed by *Enterococcus* sp, *Pediococcus* sp and *Streptococcus* sp.

Antibiotic susceptibility test results following disc diffusion method showed that all the isolates showed highest percentage of susceptibility towards chloramphenicol and highest percentage of resistance towards methicillin, streptomycin, kanamycin and amikacin, in comparison to the 12 antibiotics tested. The MIC was performed of 25 *Lactobacillus* isolates with antibiotics streptomycin, vancomycin and chlorampenicol as majority of the isolates belonged to *Lactobacillus* sp. In the future studies MIC of antibiotics for all these isolates can be carried out.

We get a general view that resistance in LAB to antibiotics could be independent of exposure to antimicrobial agents. In our study we observed, some isolates from frequent traditional medicine users (who claimed they have hardly consumed allopathic medicines/ antibiotics) were resistance towards those antibiotics which generally inhibits the growth of bacteria. The other reason for resistance could be the transmission of resistant bacteria from food sources also LAB are naturally resistant to many antibiotics.

According to the resistance pattern observed, we can conclude that majority of the isolates showed intrinsic resistance. The resistance shown by some isolates to which generally susceptibility is observed; could be due to the acquiring of resistance genes by bacteria from food sources or other environmental sources. However, we could not identify the actual resistance mechanism through molecular methods, and in addition MIC of all the isolates with desired antibiotics was not performed.

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Annexure

Annexure I

STUDY ON LACTIC ACID BACTERIA OF HUMAN GUT QUESTIONNAIRE BASED FEEDBACK FORM

Demographics

1.**Gender** - Male / Female

2. **Age (yrs.)**-(15-30) / (31-45) / (46-60) / (61-75) / (76-90) / (> 90) _____yrs old.

3.**Population type** - Rural /Urban

Name of place_____

4.**District** -North / South /East /West

General information

6. **Height** _____ **ft Weight** _____ **kg**

7. Profession
Government employee /Private sector employee
Farmer /Small self-owned business /others

8.Community –SCs / STs / OBCs/ General
Treatment preferences (Allopathic / Traditional)

9. Use of antibiotics
(Never / Occasional / Regular)

10. Use of pain-killers (Never / Occasional / Regular)

11. When was last antibiotic last used?
(Within last 24hrs/A week ago/a month ago/3 months ago / 6th month ago/1 yrs/<1 yrs)

12. Please mention frequency
(Ones in day / twice in a day / thrice a day)

If under mediation what drug are you taking

_____

_____

Dietary habits

13 Frequency of diet intake(per day) - twice /Thrice

14.Major dietary composition
Vegetarian / Non-vegetarian /vegan

15 If non-vegetarians frequency of meat consumption
Daily /weekly /fortnight/month

Meat preferred - Chicken / Mutton / fish / buff/ beef/pork

16.Diet Type (mostly taken) - Cooked / Un-cooked / Boiled / Steamed / Canned / Cafeteria style /raw/ fibrous

17. Consumption of spicy food/chili- yes / no

Food consumed within last 24hrs _____

_____

_____

Diet taken for breakfast/lunch /dinner – Heavy /light

Food components (rice/roti/vegetable/pulses/meat/fish/fruits)

_____

_____

_____

Lunch=heavy/light-

_____

_____

Dinner = heavy/light-_____

_____

_____

History of Infection/diseases

General
Heart diseases / Arthritis / Depression /Malaria

Oral
Oral ulcers / Plaque / Bleeding gums

Dermal
Leprosy / Eczema / Candida /Ringworm/Athlete's Foot / Acne

Respiratory
Bronchitis / Tuberculosis / Pneumonia/ Brucellosis / Whooping Cough / Asthma/ Influenza

Gastro-intestinal tract
Amoebiasis / Peptic ulcer /Cholera / Food poisoning /Typhiod fever, enterocolitis, dysentery, diarrhea

Liver-
Cirrhosis / Hepatitis

STDs
Gonorrhoea / Syphilis / Chlamydiasis / HIV

Eye/Ear-
Conjunctivitis /Otitis (middle ear)

Chronic disease
Diabetes/high blood pressure / cancer

Have you ever visited hospital for diagnosis?
Vidal test/ culture test .
Yes / no

Field report

- Type of sample observed
- Color of the sample observed
- Nature - dry / wet / sticky
- Frequency of defecate per day

18.Food source – own farm / market / both

19. consumption of fermented food-yes or no, if yes then tick
(gundruk /kinema / daihi / churpi / dry meats/karyong)

20. **consumption of fruits**
Yes/ no (source--

_____

21 .Water source
Government provided /Natural spring/river /Not known

22.Water consumed Raw /Boiled /Filtered /Purified

23. Health supplements like (Horlicks) / milk or beverage like tea taken with the last 24 hrs
Yes/ no-

_____

Social/Lifestyle behavior

Smoking Yes-Filtered cigarettes / Yes-Non-filtered cigarettes /No /Quit

Alcohol consumption Yes / No

Frequency of alcohol consumption
Daily /Weekly /Occasionally

Preferred alcoholic beverage
(Wine / Brandy / Beer / Rum / Whisky / Vodka Gin /Home brew)

House type Hut /Cottage /Concrete

Appropriate sanitary/ventilation conditions
Yes / No

Cooking infrastructure (Hygienic / un hygienic)

Domesticated animals
Dogs / Cat / Birds / Cows / Swine / Goat

Annexure II

Consent Form

The study entitled "To study the Antibiotic Susceptibility Pattern of isolates of Lactic Acid Bacteria from Gut of healthy individuals" is purely a scientific study conducted by Nilu Pradhan for M.Phil Dissertation under the supervision of Dr. Buddhiman Tamang, Department of Microbiology, Sikkim University.

The main objective of the study is to enumerate, isolate and perform the antibiotic susceptibility patternsof Lactic Acid Bacteria of healthy individuals from fecal samples.

The researchers have provided all the information related to this study. As per the information provides them there would be no harm to donor's health. Therefore, I would voluntarily participate in this study by providing relevant information and my faecal (sample) for the study.

Signature of the Donor

Dated:

Annexure III

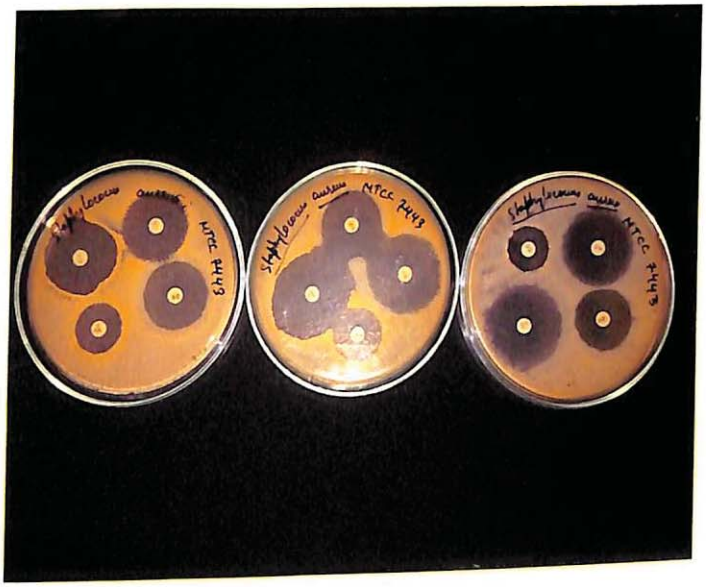
Zone Size Interpretative Chart for Antibiotics as per CLSI (2012).

Antimicrobial agent	Symbol	Disc content	R mm or less	I mm	S mm or more	Quality Control Limits	
						E.coli ATCC 25922	*S.aureus* ATCC 25923
Amikacin	AK	30mcg	14	15-16	17	19-26	20-26
Ampicillin	AMP	10mcg	13	14-16	17	16-22	_
Chloramphenicol	C	30mcg	_	_	_	21-27	19-26
Clindamycin	CD	10 mcg	_	_	_	_	28-34
Erythromycin	E	15mcg	13	14-22	23		22-30
Kanamycin	K	5mcg	_	_	-	16-22	19-26
Methicillin	MET	5mcg	9	10-13	14	-	17-22
Norfloxacin	NX	10mcg	_	_	_	28-35	17-28
Penicillin G	P	10mcg	_	_	_	_	26-37
Streptomycin	S	10mcg	_	_	_	12-22	_
Tetracycline	TE	30mcg	_	_	_	18-25	24-30
Vancomycin	VA	30mcg	_	-	_	_	-

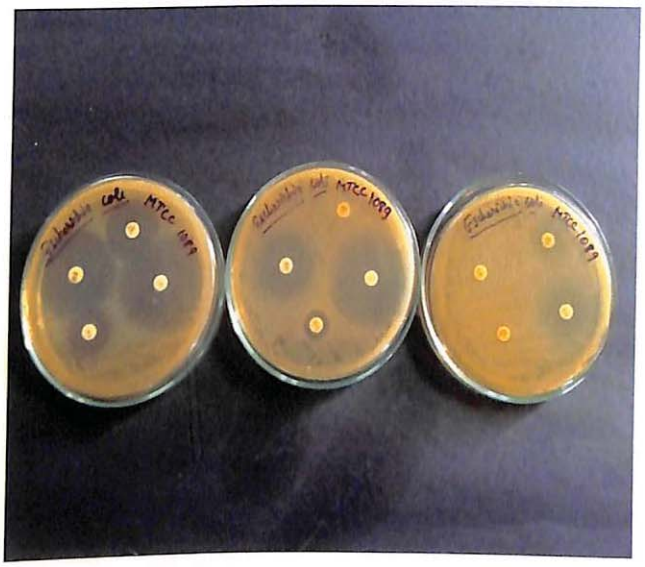
Note: R= Resistance, I= Intermediate, S= Susceptible, zone of inhibition in mm.

Photographs

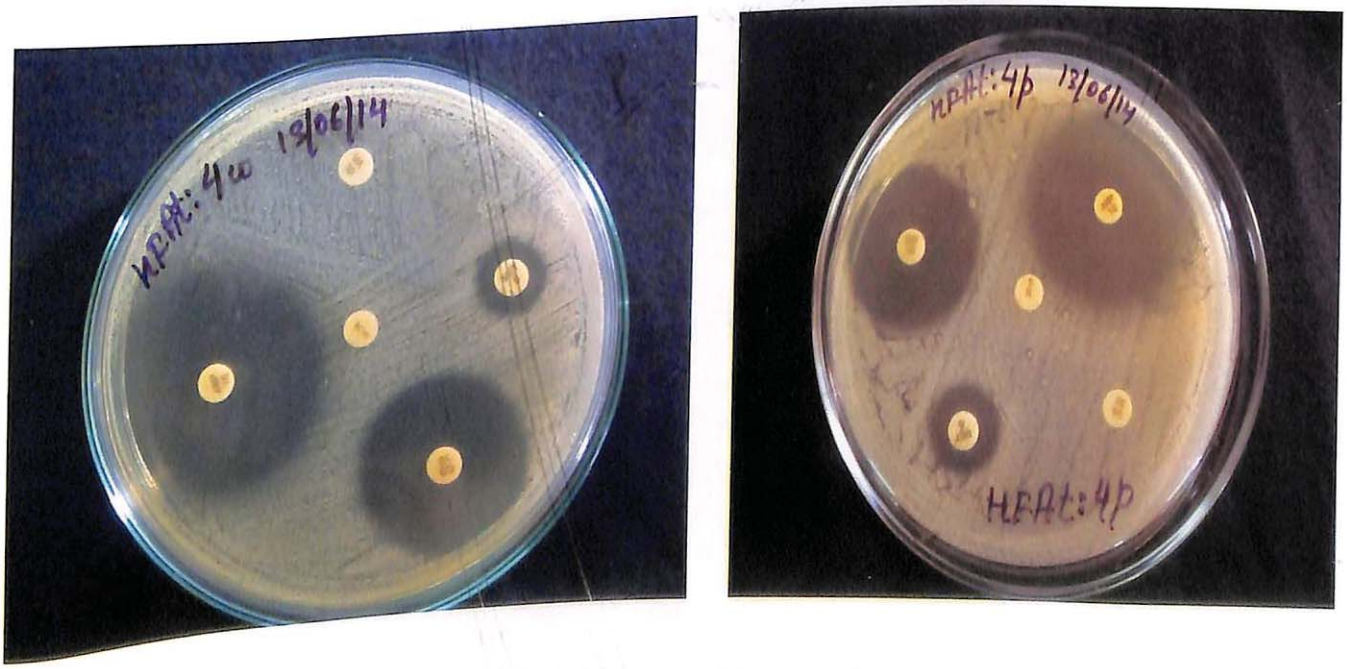
PHOTOGRAPHS



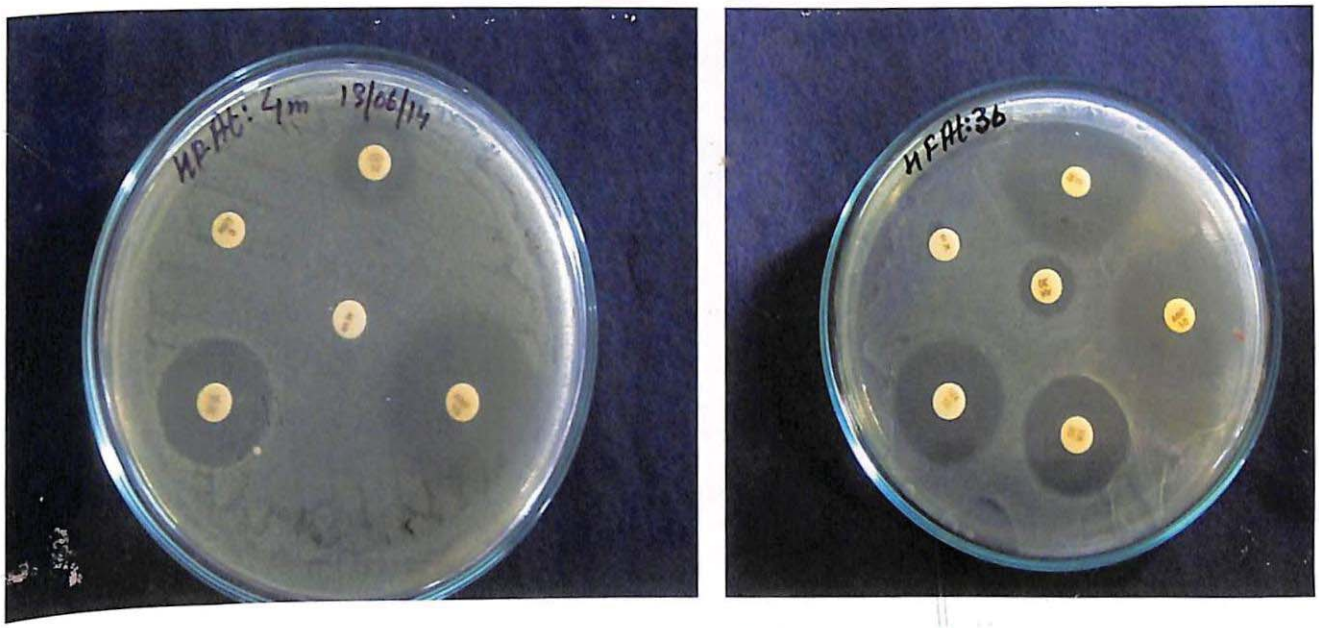
Pic 1. Disc Diffusion Test of control strain *S. aureus* MTCC 7443.



Pic 2. Disc Diffusion Test of control strain *E. coli* MTCC1089.



Pic 3. Disc Diffusion test of *Lactobacillus* isolates.



Pic 4. Disc Diffusion test of *Pediococcus* isolates

Pic 5. Disc Diffusion test of *Enterococcus* isolate