1. Abstract

Hypercholesterolemia is defined as high cholesterol level in blood serum and usually associated with the cardiovascular diseases. The conventional treatments are costly and have reported adverse effects. Several studies reported that *Lactobacillu*s strains have the ability to decrease cholesterol level during consumption. So probiotics could be an alternative treatment. The objective of this study was to investigate the safety, survival and cholesterol lowering potential of previously isolated *Lactobacillus* species in vitro and in vivo using High Fat Diet rat model. The *L .rhamnosus (I-2 & I-12), L. salvarius (I-7a) and Lactobacillus* (I-17) survived and colonized the GIT. The rats were gaining weight and no symptoms of disease were shown highlighting the safety of the strains. Two out of four strains i.e. *L.rhamnosus (I-2) and Lactobacillus (I-17) showed* significant BSH activity with maximum cholesterol assimilation in the broth medium. Furthermore, administration of these probiotic dosages to the rats fed with High Fat Diet resulted in significant reduction in cholesterol, better LDL and HDL ratios at the end of 30 days trial as compared to control and statin fed group. These strains have variability in cholesterol reduction ability. Our study showed that both BSH positive isolates i.e. *L.rhamnosus (I-2) and Lactobacillus spp. (I-17)* have also comparable *in vivo* results as compared to the commercial available drug i.e. statins . The results of the study suggest that these *Lactobacillus* strains can be used as potential probiotic strains for cholesterol reduction. These isolates can be used as an alternative treatment for serum cholesterol reduction. However clinical trials are required for conclusive verdict for its use in cholesterol treatment.

.

.

2. Introduction

2.1 Hypercholesterolemia:

Hypercholesterolemia is basically a condition characterized by presence of high cholesterol and lipoproteins in the blood serum. Cholesterol is a waxy substance like fat produced in the body and obtained from animal and plants food sources. Cholesterol is an important substance need by human body for production of hormones, proteins and fat digestion compounds(Anandharaj et al, 2014). Cholesterol is of two types and is refereed as Bad and Good cholesterol. Lipoproteins are the substances that carry cholesterol in the blood through the body. HDL or high density lipoproteins are referred as good cholesterol and are associated to remove cholesterol from the body(Singh et al., 2015). High levels of HDL are associated with low heart diseases. LDL/ low density lipoproteins are associated to carry cholesterol in the blood ((Charbonneau & Healy, 2005). High levels of LDL are the major cause of elevated blood cholesterol.

2.2 Causes of Hypercholesterolemia:

Hypercholesterolemia may be cause due to different factors. Some are hereditary and some are related to our unhealthy life style. Some of major causes are mentioned below with their problems in table 1.

2.3 Cardiovascular diseases by Hypercholesterolemia:

Hypercholesterolemia is widely accepted as a leading factor for developing cardiovascular diseases i.e. coronary artery diseases, atherosclerosis and stroke etc. In this condition, LDL is deposited in blood vessels (coronary arteries). These depositions harden the artery walls and narrow the path by clumping of cholesterol as fig 1. This situation can easily restrict the blood flow to the heart. This blockage causes angina (chest pain) and increases risk of having heart attack(Charbonneau & Healy, 2005)

Table 1: Causes of Hypercholesterolemia:

Factors	Problem	Reference
Genetic factors	Mutation in genes LDLR, APOB, LALRAP1 & PCSK1 cause hypercholesterolemia	(Charbonneau & Healy, 2005)
Diet	Easting too much saturated and trans fat, food with cholesterol like red meat & full fat milk products.	(Formulas, 2008)
Smoking	It basically lowers the HDL level and Accumulation of fats by the damage of blood vessels.	(Matos et al., 2005)
Diabetes	It basically damages the arteries lining.	(Johansen, 1990)
Obesity	Body Mass index higher than 30 increase the risk of cholesterol	(Formulas, 2008) (Charbonneau & Healy, 2005)
Others like sedimentary life style, alcohol and kidney issue	Lower the HDL level	(Charbonneau & Healy, 2005)

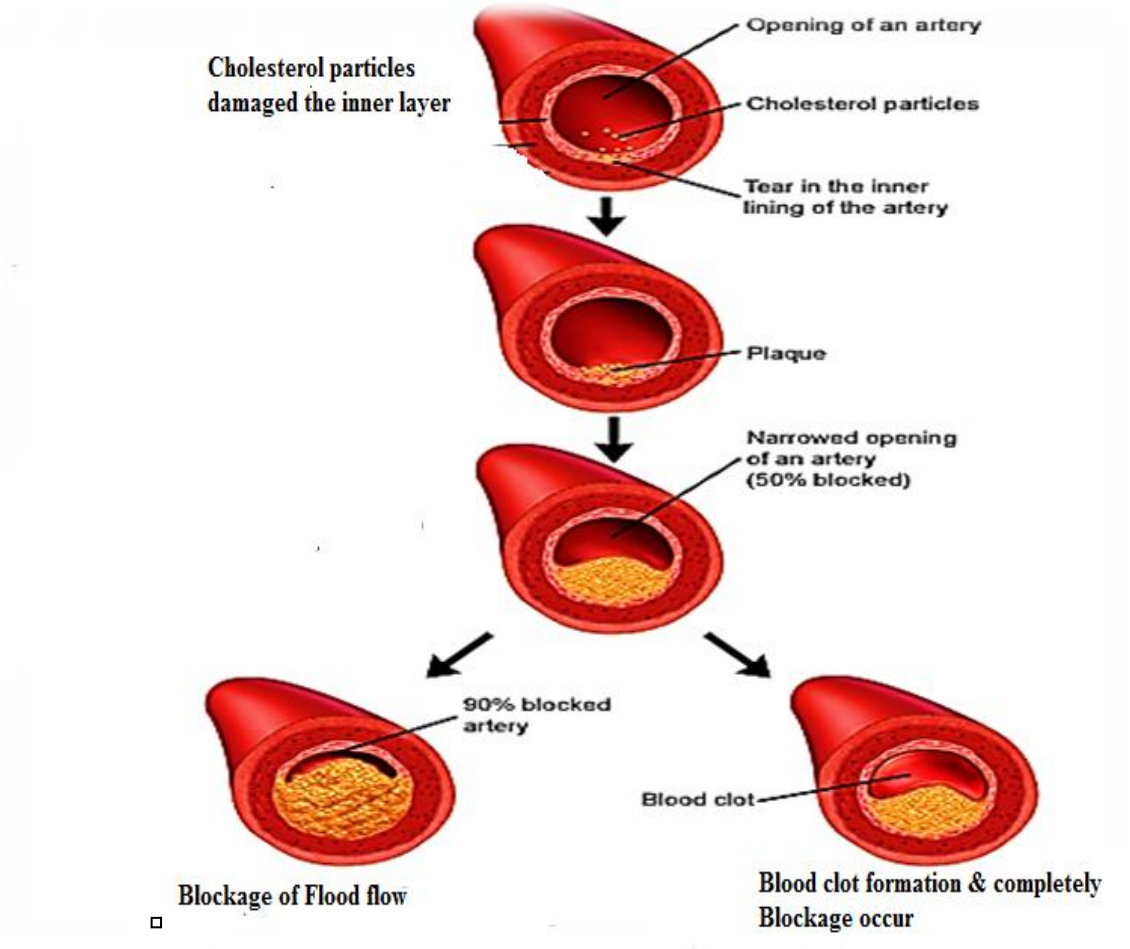


Figure 1: Blockage of arteries due to Cholesterol Assimilation

2.4 Prevalence:

According to the WHO, Cardiovascular diseases are considered as the main global cause of mortality and morbidity. Therefore it became a hot debate for scientific focus both for industrial and academic sectors(Hu, Wang, Li, Jin, & Wang, 2013). By 2030, WHO has predicted that 23.6 million (M) people will affect/die due to this and it will remain the leading cause of mortality. In 2014 report of Who, it was stated that 17.5M people died due to cardiovascular diseases (CVDs) (Nocianitri et al., 2017). It was about the 31% of total death case of the world. Out of these 7.4 were due to coronary diseases and other 6.7 M were by stroke (WHO, 2014). From 1999 to 2003,In Western and Central Eastern Europe due to hypercholesterolemia the heart attack were

approximately 40-45% (Yusuf et al., 2004). In the US about 33% death is due to heart diseases and about 84 million people are suffering from CVDs(Of, Death, & To, 2018). In the report of British heart foundation, 7 million people are suffering from CVDs("UK Factsheet," 2018).

According to another report it was stated that if you 1% increase in blood cholesterol the chances of heart diseases is 3 times more than the normal profile one. Pakistan is on the highest risk of cardiovascular diseases. In 2007 17M deaths were due to cardiovascular diseases. In 2018, a medical study survey report, it was stated that 8 person per hour die in Pakistan due to heart attack and about 40% deaths are due to cardiovascular diseases. And 66% of heart diseases in Pakistan are due to high cholesterol (Zubair et al., 2018).

2.5 Conventional Treatment:

There are many conventional treatments which are mostly used for the cure of hypercholesterolemia. The medication which globally available for the cure of this high level cholesterol are are medications, vitamins, enzymes inhibitors and food supplements as shown in table 2. All these medications need to be used in combination or with exercise.

Conventional treatment of hypercholesterolemia has many adverse side effects and costly as discussed in table 3. Other disadvantages includes acceptance of consumer, availability and their effectiveness (Koslik, Meskimen, & Golomb, 2017).

Table 2: Available treatments of Hypercholesterolemia:

Medications	Statins, it is available in atorvastatin, simvastatin and rosuvastatin. All of these are useful for the inhibition of lever enzyme production that helps in the production of cholesterol (Han et al., 2017).
Vitamins	Niacin. It is vitamin B, which is used to reduce the triglycerides and increase the Good cholesterol/HDL content (RIVIN, 1962)
Enzyme Inhibitors & Lipid Reducing Polymer	Ezetimble is a substance that helps to inhibits adsorption of cholesterol (mostly come from our regular diet) from the gut. Colestipol, it is basically a bile acid sequestrant used for lowering of Bad cholesterol or LDL from the blood(Pakes Brogden RN, Heel RC, Speight TM, Avery GS., 1981)\.
Plant & Animal Supplements	Plant stanols and sterols, it is natural substance like cholesterol but produced by plants instead of animals. Human body cannot absorb plant sterols. Due to same structure sterols compete with animal cholesterol, as a result human body grabs extra LDL from the blood and caused the LDL cholesterol reduction (AbuMweis, Barake, & Jones, 2008). Fish oil, it is omega-3 fatty acid poly unsaturated fat, which is used to reduce triglycerides. It basically reduce the production of triglycerides in liver (Singer, 2011):(Weitz, Weintraub, Fisher, & Schwartzbard, 2011).
Dietary Supplements	Dietary Supplements (flavonoid and polyphenols), Garlic, leaf extract, guggul extract. Grape polyphenols, tea catechins, hawthorn fruit, buckwheat and policosanol are the compounds having cholesterol lowering ability but their studies are small and poorly designed (Plaque, n.d.)

Table 3: Disadvantages of conventional medicines:

Treatment	Drawback	Reference
Medications	• Muscle pain • Liver damage • Increased blood sugar • Neurological side effects • Miscarriage	(Ramkumar et al, 2016), (Lee et al, 1996) (Koslik et al., 2017)
Enzyme Inhibitors & Polymers	• Severe constipation • Black stool • Chest pain • Breathing issue • Stomach pain • Swallowing	(Shah et al, 2013) (Defined & Facts, n.d.) (Pakes et al, 1981)
Vitamins	• Nausea • Uric acid • Drowsiness • itching • Headache • Insomnia • Gas	(Meyer-Ficca et al, 2016) (W.-L et al, 2013) (Koslik et al., 2017)
Dietary & plant supplements	• Weight gain • Itching • Constipation • Chest burn • Bleeding • Stomach pain	(Lee et al., 1996) (Modifica- & Cyril, 2008) (AbuMweis et al., 2008)

These side effects make these conventional medications or treatment less effective and dangerous for the human health (Koslik et al., 2017)(Meyer-Ficca, M & Kirkland, 2016). So

there is an urgent desire for the new therapies to cure hypercholesterolemia. This problem has led scientist to discover new therapies with less side effects with better results (Ramkumar et al., 2016) .

2.6 Alternative strategy:

Alternative of these conventional treatments is the consumption of probiotics, which have cholesterol reduction ability and can decrease blood cholesterol level (Chen et al., 2011). According to Who definition “probiotics are live micro-organisms when administrated inadequate amount confer health benefits” (D. Song, Ibrahim, & Hayek, 2012). Almost all probiotics are lactic acid bacteria (LAB) and are regarded as safe by FAO and WHO (Anandharaj et al., 2014).

The usage of probiotics for different diseases like diarrhea, ulcers and stomach problems are not new and are commercially available for these diseases (Ziemer & Gibson, 1998). According to the recent studies, probiotics have many benefits and role in treatment of different diseases, which are listed below:

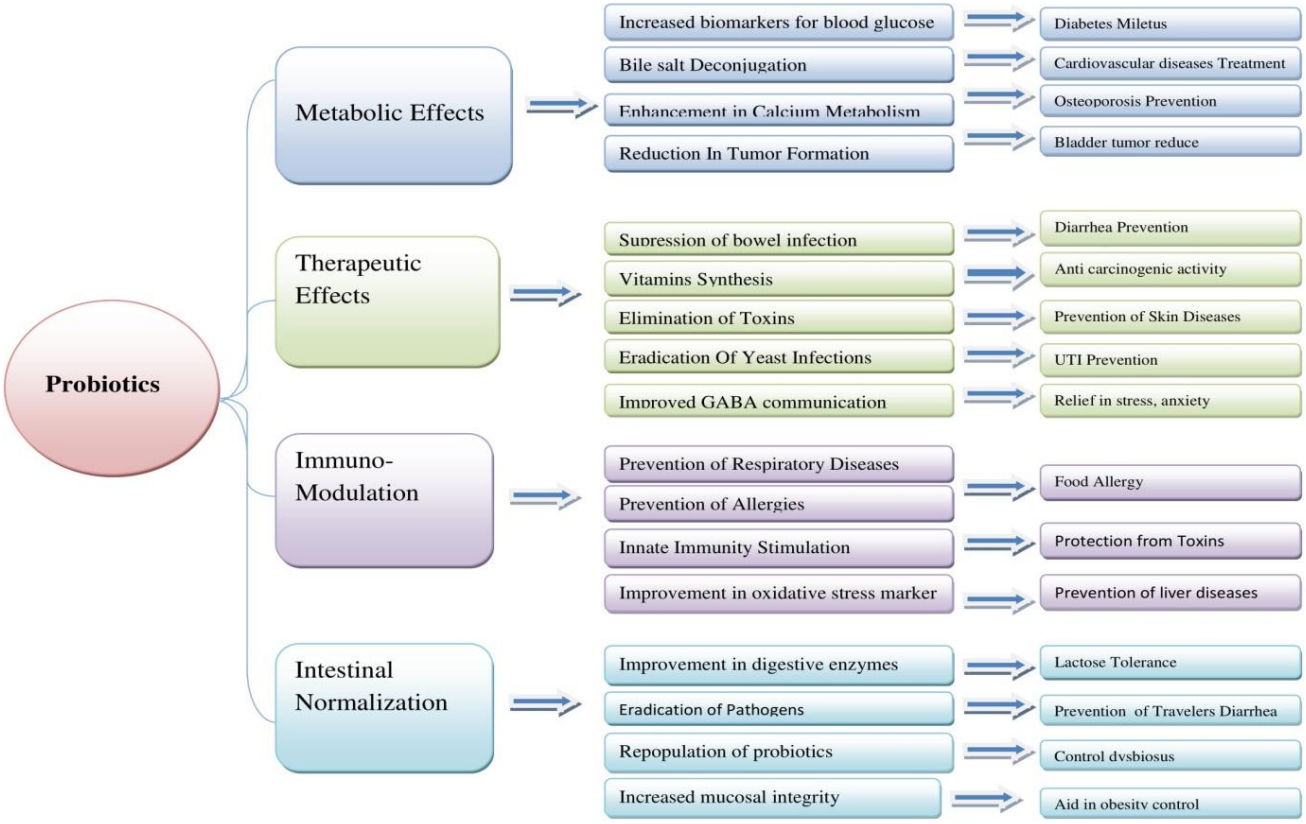


Figure 2: List of main benefits by Probiotics

Including all of above treatment of hypercholesterolemia is one of the probiotics benefits (Tomaro-duchesneau et al., 2014):(Al-awwad, Takruri, & Yamani, 2014)(Singh et al., 2015). This ability of probiotics has been evaluated in some LAB species *(Lactobacillus, Streptococcus & Enterococcus) And Bifidobacterium species* (Pereira & Gibson, 2002). According to different studies, it has stated that the administration of probiotics can reduce the blood cholesterol level up to 45% (Abd et al., 2016). On the behalf of different studies we can use this property in the treatment of hypercholesterolemia.

2.7 Mechanism of Probiotics for cholesterol reduction:

For the support of this cholesterol reduction property, different mechanisms for cholesterol reduction of probiotics have been proposed. These include different mechanisms which are given below:

- Cholesterol Assimilation by live cells (Nguyen, Kang, & Lee, 2007)
- Incorporation of cholesterol into cellular membranes (Lye et al,, 2010)
- Attachment on cellular surfaces by the interaction of exopolysaccharides(Lavanya et al, 2011)
- Conversion into short chain Coprostanol (Lye et al., 2010)
- Bile salt hydrolase activity(Liong & Shah, 2005)\

Evaluation of the cholesterol lowering potential of the probiotics for their use in treatment of hypercholesterolemia, their characterization, safety and survival in the gut and their in-vitro potential are very essential. These help in the strain identifications that are able to decrease serum cholesterol (Lavanya et al., 2011).

2.8 Functional Foods & Probiotics products:

Many products are commercially available that helps in different diseases. These products are easily available and have a large impact on the market share. The market valued share in 2017 of probiotics was USD 40 Billion and growing during 2018-1024 at the rate of 7.35 CAGR (23.93 billion). It is estimated by the experts that the probiotics revenue will be approximately USD 137 billion. The main markets of these probiotics and their products are Europe and Asia pacific.

Table 4: Popular probiotics products:

Product	**Concentration of bacteria**	**Company**
PRO-Kids	(4.4 billion cfu)	Hyper-biotics
BlueBiotics	Containing mixture of *Lactobacillus* species with *S.Boulardii* (61 billion cfu)	Blue Biology
Ultimate flora	12 strains of *Lactobacillus and Bifidobacterium* (50 billion	Renew life

	cfu)	
Ultimate Care	34 Probiotics strains (100 billion cfu)	RAW PROBIOTICS
PRO-25	13 probiotics strains (25 billion cfu)	Vitamin Bounty
Mega Flora	14 probiotics strains(20 billion cfu)	Mega Food
NEXA Biotic	23 probiotic Strains (34.5 billion cfu)	DrFormulas
PB 8	*L.acidophilus* (14 billion cfu)	NUTRITION NOW
Digest Gold	5 probiotics strains (15 billion cfu)	ENZY MEDICA
Yakult	*L.paracasei* fermented milk	Yakult.

Adapted from (Consumer Report, 2018).

In this present study, four previously isolated *Lactobacillus* strains from different food sources with the best probiotic potential were selected. The best probiotic potential included tolerance to acid, bile and phenol and ability to adhere to small and large intestine. These four have not any history and food product usage and cannot referred as safe or GRAS. So it was necessary to conduct safety and survival assessment. For this safety and survival evaluation of these four strains was conducted on rat model. Then we test in vitro cholesterol lowering ability of these four strains compared with commercially available probiotic strain. On the basis of in-vitro cholesterol reducing results of these four strains, three were selected and evaluated their cholesterol reduction ability in rat model.

3. Objectives

- In vitro cholesterol assimilation of the selected Lab strains.
- Assessment of safety and survival of the strains in the GIT using rat model.
- Evaluation of cholesterol reducing potential of selected strains in rat model.

4. Literature review

4.1 Hypercholesterolemia:

Hypercholesterolemia or dyslipidemia is the medical problem in which cholesterol, lipoproteins and blood lipids are present in high level than normal. Different studies stated that the high cholesterol level is associated with the cardiovascular diseases, which are the main cause of mortality in the developing countries. The diseases associated with high cholesterol level are chronic diseases like heart failure, stroke, ischemic heart diseases and kidney problems. According to clinical trials, it was revealed that increase in 1mmol in cholesterol level, increased the 35% risk of getting CVDs (Singh et al, 2015). Production of arterial plaques is due to the oxidation of cholesterol, which is also a major cause of CVDs as shown in Fig 1. Hypercholesterolemic person has 3x more chances of heart attack than a normal person. Studies revealed that hypercholesterolemia also lead to hypertension; it is a condition of high blood pressure. Lower level of HDL and high level of triglycerides have been found to be a main reason of high blood pressure in the individuals. It is also verified by the different studies that if cholesterol exceed 6.4 mmol/L then there is significantly increase in blood pressure(Lye et al, 2010) .

It has been predicted by WHO, that by 2030, the main cause of death globally would be due to CVDs, affecting approx. 24 M people. By WHO, definition of unhealthy diets is that those diets which have high fats, free sugar and salts and low carbohydrates (Nocianitri et al., 2017). And the unhealthy diets are also the main cause of cardiovascular diseases as discussed in table 1. Many of the studies suggest that probiotics decrease the risk of heart attack and CVDs(Saikia et al., 2017).

The usage of animals and humans models has been emphasized over the years to evaluate cholesterol assimilation by different probiotics strain. Human studies have already shown promising results while new strains have been evaluated for their Hypercholesterolemic potential. In many trial animals like rats, pigs, mice, guinea pigs, hamster, poultry and monkey have been used as a model because they have similarities cholesterol metabolism, distribution of plasma protein and enzymes regulations. They also share same physiology, nutrient consumption and digestive anatomy, metabolic and bio-availability processes with human. This makes them

ideal candidate for research purposes. The positive results shown in animal model state the same potential in humans as obtained from selected animals(Kumar et al., 2012).

In early 1974, two scientists Mann and Spoerry discovered the cholesterol assimilation benefit of fermented milk consumed by Massai. (tribe). More studies have suggested the administration of probiotics to reduce cholesterol.Study Ha et al. (2006) reported that consumption of *L.plantarum CK102* was assessed to lower the cholesterol level, LDL and triglycerides in rats by 28, 29 and 62%. Similarly it was also found that *L.plantarum KCTC 3928* reduced the LDL level by 33% and HDL increase by 42% respectively(Liang et al, 2016). Many studies on humans on the benefits of probiotics showed different results on blood lipid profile. Naruszewics et al. (2002) and Bertolami et al. (1999) stated that probiotics could help in betterment of blood lipid profile. Lewis (2005) found that consumption *of L.rhamnosus LC705* reduce the cholesterol level from the blood serum. Study by Xiao et al.(2005) found that *B.lognum BL1* showed significant decrease in LDL, Cholesterol and triglycerides and approx. 15 % increase in HDL. Trial conducted on the reduction of cholesterol stated that *L. reuteri CRL 1098* reduced 38% cholesterol when administrated to the animal model for 15 days.it also caused the 40% reduction in triglycerides and 20% increase in HDL ratio(Ooi & Liong, 2010). In a study, administration of *L. plantarum PH04* to Hypercholesterolemic rats for 14 days. It was found that *L.plantarum PH04* reduces the total cholesterol by7% and triglycerides by 13%(Lee et al, 2007) . In another trail albino rats fed with *B. longum Bb-46* for 35 days. It significantly reduced the cholesterol concentration by 50% and triglycerides by 52% and LDL by 57%(Pereira & Gibson, 2002). Another study showed that rats fed with *L. acidophilus* fermented rice for 28 days. It was found that significant decrease in complete blood lipid profile by 23%(Chen et al, 2011).

Study on human showed that consumption of 200g yogurt containing L. acidophilus L1 daily after one meal contributed to reduction of blood lipid profile. In another human trail by Xieo et al, assessed that *B.lognum BL1* also affect the lipid profile on regular consumption. It was also observed an increase of 18% in HDL concentration. However there are studies in which probiotics are totally failed to reduce blood serum. This need more research to evaluate their potential (Tomaro-duchesneau et al., 2014)

.

4.2 Conventional treatment:

The conventional treatments to reduce blood cholesterol are drugs therapies and different dietary supplements. These treatments have very adverse side effects reported that leading to seeking alternative strategies like weight loss, exercise and diet (Bilznakov. 2002). These treatments are also very costly and adds more expenses to the patients, as these medications are taken for whole life (Miremedi et al, 2014).

Studies stated that adverse effects associated with statin are muscle pain, myalgia, hypothyroidism, liver problems and increase sugar level as in table 3 (Ramkumar et al,2016). In another study use of niacin to reduce cholesterol stated that the long term usage of niacin cause some gas, itching, severe chest burning, vomiting and uric acid issues. According to studies and different case reports by Koslik et al (2017) stated that effects of the conventional treatments on different are so serious that they can cause severe muscle pain, swallowing problems, insomia, breathing issues and stomach disorders (Koslik et al., 2017).

Some of the human trials have been shown that food supplements used to cure high cholesterol leads to severe side effects that include bleeding, weight gain and GIT related issues.

4.3 Probiotics:

The utilization of probiotic microorganism bounces back into prior time before the discovery of microbes. The bacteria and yeasts which are responsible for the fermentation process were first recognized by Louis Pasteur, yet he was not ready to demonstrate any clear association of these microbes with health benefits. Scientifically, the foundation for using live microorganism for the treatment and prevention of infections emerged toward the beginning of the twentieth century in 1907, with the hypothesis of Elie Metchnikoff, who previously worked with Pasteur in 1860s(Anadon et al.2016). His hypothesis states that by diminishing or exchanging the amount of putrefactive bacteria residing in the gut with lactic acid bacteria can extend lifespan and stabilize bowel health. By the end of 20th century, the term probiotics was originated to reflect the idea of Metchnikoff. Elie Metchnikoff is considered as the grandfather of probiotics. The term “Probiotics” was first used by Lilly and Stillwell in 1965 to signify, ‘‘substances which are released by one microorganism which inspire the growth of another microorganism.” Nine years after the fact, Parker characterized probiotics as “The organisms that

are used to balance the intestinal micro flora" (Gogineni, 2013). After Fifteen years, Fuller suggested that probiotics are 'living microorganisms that positively affects host body by improvement in the equilibrium of microorganisms. After this, Salminen et al. defined probiotic microorganisms as 'living bacteria present in the food which provides benefit to health' (Status, 1999).

The WHO and United Nations FAO have already declared the usage of probiotic for the human health benefits as no pathogenic factors and virulence factor have been reported and regarded probiotics safe for human health (Cordier, 2008). Since ancient times, LAB has been use in the production of fermented dairy products. However the applications of probiotics in agriculture, aquaculture, animals and vegetarian food products have not gained much recognition (Song et al, 2012). The introduction of probiotics into new application has attracted the scientific attentions. These probiotics not only work as a starter culture in fermentation processes but also have a vital role in digestive mechanism that benefits to human and animal health (Song et al, 2012).

According to various studies probiotics deliver health benefits to humans by performing different procedures. The function performed by probiotics include lactose intolerance, mucosal immunity modulation, balance of gut micro-flora, reduction in dermatitis manifestations, diarrhea, constipation, Crohn's diseases anti-cancer effects, removal of UTI infections and cholesterol reduction in blood serum (Chávez-tapia et al, 2015) (Huey-shi Lye et al,2009).

Besides all advantages the cholesterol reduction potential has great importance related to the CVDs maintenance (FAO/WHO, 2002). According to studies of Mann and Spoerry were stated that probiotics could work as a biological or bio-therapeutic agents. Probiotics with bile salt hydrolase activity have been reported to reduce cholesterol levels. This potential of probiotics have also been proved in vitro test such as liquid media and also in in-vitro trials of animals and humans (Tomaro-duchesneau et al., 2014). Study of Pereira and Gibson have stated that the probiotics which have shown promising cholesterol reduction potential are *Bifidobacterium infantis, Enterococcus gallinarium, Enterococcus faecium, Enterococcus durans, Enterococcus faecalis, Streptococcus bayis, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus reuteri and Lactobacillus Acidophilus N5*(Pereira & Gibson, 2002).

The selection of potential probiotic strain is a real challenge because the strain should be able to survive harsh environment of GIT(Singh et al., 2015).

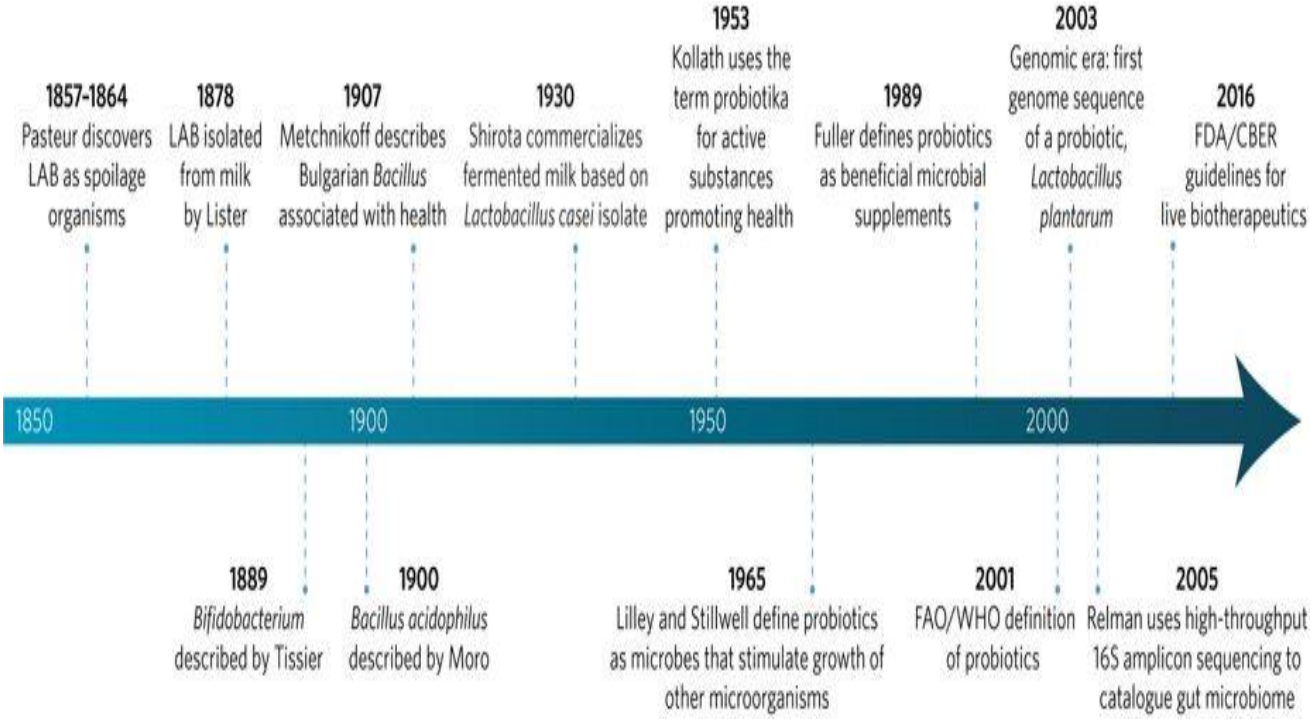


Figure 3: Time line of probiotics Adapted from Hill C et al,(2017)

4.3.1 Taxonomy of Probiotics:

Lactobacillus and *Bifidobacterium* are the two most significant genera in the field of probiotics. Some other genera including *Enterococcus, Pediococcus, Lactococcus, Saccharomyces, Streptococcus, Bacillus* and *Escherichia coli* likewise contain the fascinating species that can be utilized as probiotics. Member of genus *Lactobacillus*, *Lactococcus,* and *Leuconostoc* (less commonly) that are being used as Probiotics, are called Firmicutes and are assigned as lactic acid bacteria (LAB) collectively. Other probiotic microorganisms that are being used have a place with the class Bifidobacterium, falls in the phylum Actinobacteria. These genera entirely comprise the species that do not cause illness upon colonization in the intestine, , in spite of the fact that exemptions do exists example includes, *Bifidobacterium dentium* that cause dental disease, otherwise these are more generally the members of normal

oral flora.. *Lactobacilli* are the normal flora of the human gut, especially in the colon approximately 9 log CFU/g. *Bifidobacterium* and *Lactobacillus* are the two most widely used probiotic genera and neither of them includes any major pathogenic species. Naturally in humans *lactobacilli* resides in the oral cavity (10 3-10 4 CFU/g), the ileum (10 3-10 7 CFU/g), and the colon (10 4-10 8 CFU/g. The most commonly probiotic strains used in different food products are:

Table 5: Commonly used probiotics:

Lactobacillus	***Bifidobacterium***	**Other species**
L.acidophilus	*B.lognum*	*Streptococcus thermophilus*
L.casei	*B.animalis*	*Bacillus subtilis*
L.fermentum	*B.adolescentis*	*Sporolactobacillus inulinus*
L. johnsonii	*B.bilidum*	*Pediococcus acidilactici*
L. gasseri	*B.breve*	*P. pentoseceus*
L.reuteri	*B.lactis*	*Saccharomyces boulardii*
L.bulgaricus	*B.inlantis*	*Lactococcus lactis*
L.plantarum		*Enterococcus faecalis*
L.rhamnosis		*Escherichia coli*
L.delbruckii		*Enterococcus faecium*

(Adapted from Furrie, 2005)

4.3.2 Probiotics Market:

The global sale of product containing probiotic was US$21.6 billion in 2010 and in 2011 it achieved US$24.23 billion. The worldwide market of probiotics is probably going to reach to US$44.9 billion in the end of 2018. The probiotics sales are increasing at the rate of 7.38 CAGR. At present the greatest probiotic advertise is Asia-Pacific and is relied upon to remain the pioneer of the market. The project sale of probiotics in 2024 will be 137 Billion dollars

4.3.3 Characteristics of an ideal probiotic candidate:

While selecting a potential probiotic strain, number of aspects should in mind and selection basis should have in-vivo safety and survival assessment, able to function in the GIT environment, adhesion to intestinal lining and colonize lumen of GIT. The strain should have no antibiotic resistance genes and should be able to survive GIT transit of acid and bile stress. Strains must have viability & genetic stability with excellent technological stability so can be used in fermented food products without losing their viability and flavors and texture.

4.3.4 Acid and bile tolerance:

Probiotic bacteria are commonly provided in foods, so they begin their voyage from mouth to bring down intestinal. As for that, these bacteria should resist to the enzymes like lysozyme in the oral cavity and they should also be resistant to the digestion process in the gastro intestinal tract and stomach(Patel et al, 2012) . A study reported that, 90 min is duration from entry to release from the stomach. The stress begins in the stomach in the form of low pH, such as 2. After passing through the stomach, they enter the small intestine where there is secretion of bile In the wake of going through the brutal distressing condition, the microbe starts colonizing to the epithelium of the lower intestinal tract. In this way, the probiotic strains should tolerate acidic stress for as a minimum 90 min, resist bile acids, do attachment to the epithelial cells, and engender in the lower intestinal tract before the start of providing health benefits (Begley et al, 2005).

4.3.5 Resistance to phenol:

The characteristic of an effective probiotic is that it should maintain viability, tolerate GIT stress, do adhesion and colonization in the gut epithelium and ought to be protected upon consumption. Phenolic compounds are released by the deamination of dietary proteins and a probiotic ought to likewise have capacity to endure phenol stress (Patel et al., 2012).

4.3.6 Survival and Colonization to the GIT:

Attachment of probiotics to the intestinal walls is a very vital characteristic for the probiotics. This is because adherence of cells to the intestinal mucosa helps the bacteria in colonization and the beneficial properties of the probiotics can be imparted more efficiently if the bacteria undergo colonization. Colonization is a temporary property of the probiotic strains. They colonize the intestines only for a small period of time and are found to be absent from the fecal samples after 2 to 3 weeks of dose administration (Ouwehand et al, 2003)

Every probiotic strain is habitat specific. One probiotic might survive well in one habitat than the other. For example Bifidobacterium colonizes the intestinal mucus more efficiently and effectively than any other site of the body (Lee et al, 2009) .

The probiotics can colonize to the following microhabitats in the GIT (Fuller et al, 2012) :

1) Epithelium cells surface
2) Small intestine i.e. caecum and colon
3) Epithelium mucus
4) Intestines Lumen

Adhesion is essential for the many important physiological benefits that the probiotics impart in the living organisms. Some of the physiological processes in which probiotic colonization plays an important role are:

- Stimulation of the immune system (Toumola et al, 2001)
- Making the nutrients bioavailable (Pervez et al, 2006)
- Causing a competitive exclusion of entero-pathogens (Lee et al, 2002)

Adhesion and colonization of probiotics to the gut gives more time to the bacteria to impart their beneficial effects as it increases the retention time of bacteria in the gut. According to a previous study *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG, these two help in the treatment of diarrhea and are highly adhesive to the GIT (Salminen et al, 2006).

To study the mechanism and the ability of the probiotic strains to adhere, several models have been developed. The cell lines most commonly used for adhesion assays are Cac2 cell lines, HT-29 and HT-29 MTX cell lines. For a better understanding of the mechanism of adhesion in-vivo studies have been carried out because the cell lines cannot mimic the exact gastro-intestinal environment. Thus the exact capacity of the probiotics to adhere to the GI tract cannot be determined using cell lines. Conversely, biopsy samples of the intestines and colon mimic the real conditions and can be used as a model for adhesion assays (Toumola et al, 2001).

4.3.7 Safety of the potential probiotics:

The safety assessment of probiotic strains is an important prerequisite for using them in-vivo. Probiotic strains of *Lactobacilli* have been used safely in the past and are still in use. There are some probiotics whose safety profile has not been assessed yet but their wide use in the history can be regarded as the evidence of their safety. The considerations that are taken into account before regarding a probiotic as safe are as follows (Sanders, 2003):

1) History of safe use of the probiotics through the route of administration applied.
2) The condition of health and the immune system of the consumer.
3) Potential to transfer antibiotic resistance.
4) Association of the probiotic strain with any infections.
5) The infection develops through sensitivity of administrated antibodies
6) Production of harmful metabolic compounds or mammalian toxins

A number of Lactobacillus and Bifidobacterium species have been generally regarded as safe. Some strains of Enterococcus and Streptococcus that are used as probiotics are known to be opportunistic parasites (Salminen et al, 1998).

In addition to the source of isolation, taxonomic characterization of the microbe, complete information about the microbe and the description about its final use should also be known (Fontana et al,2013). Following properties should be known while selecting a probiotics in table 6:

Table 6: Key characteristics of a potential probiotic:

Probiotics Potential	Target	Benefits
Colonization to the GIT	Impact on GIT area or site	Strengthening the mucosal barrier, Maintenance of Gut micro-flora
Metabolites production	Anti-Microbial potential	Regulation in bowel movements
Quorum sensing characterization	Reacting and sensing to Intestinal Micro-Flora	Fighting against pathogens
Cytokines production	Inflammation reduction	Strong intestinal immune responses
Assessment of toxin binding	Toxin binding from food and diet as mycotoxins & heavy metals	Intestinal integrity increased
Gene expression	Switch ON & OFF gene target	Health effects with gene expressions
Safety potential	No antibiotic resistance gene, production anti-inflammatory cytokines, non-invasive cell lines models.	Proven health benefits Safe human usage Functional food usage

Adapted from (Gueimonde & Salminen, 2006:Soccol et al., 2013)

4.4 Exopolysaccharides (EPS) Production:

EPSs are also defined as carbohydrates polymers that are associated with capsule formation on cellular surfaces and extracellular mucus secretion. Some of probiotics strains have EPSs production in a very huge amount that can be used in industrial level in form of curdian, dextran and xanthan gums (Silva et al, 2006). They are used in different suspension and films production and also as gelling agent, emulsifiers, lubricants, stabilizers, thickeners and coagulants. They have also used in food industry for the alteration of texture, elasticity and viscosity of food (Rottata et al, 2009).

Several *Lactobacilli*, together with *L. delbrueckii subsp. Bulgaricus* is equipped for supply of exo-polysaccharides that either encapsulates the bacterial cell or they are excreted into the extracellular surroundings. The usage of EPS-producing starter cultures, including *L. delbrueckii subsp. Bulgaricus*, is one way of upgrading the consistencies of yogurt throughout fabricate. Moreover, the benefits of EPS production by *Lactobacillus* have anti-tumor, immune-modulating, anti-ulcer and cholesterol lowering profile. They secure the bacteria against phagocytosis, desiccation, phage attack, toxic compounds or antibiotics, osmotic stress and EPS also helps in adhesion for a longer duration. Until now 30 EPS producing species of *Lactobacillus* have been identified. The optimum producing EPSs strains are *L.casei, L.acidophilus, L. curvatus, L. helveticus, L.plantarum and L.brevis* (Polak-Berecka et al, 2013)(Badel et al., 2011).

4.5 Mechanism of cholesterol reduction:

Probiotics use different mechanism for the cholesterol reduction but the exact mechanism is still undefined. Different mechanisms have been purposed for cholesterol reductions are given below:

- Cholesterol Assimilation by live cells (Nguyen et al., 2007)
- Incorporation of cholesterol into cellular membranes (Lye et al,, 2010)
- Attachment on cellular surfaces by the interaction of exopolysaccharides(Lavanya et al, 2011)
- Conversion into short chain Coprostanol (H Lye et al., 2010)
- Bile salt hydrolase activity(Liong & Shah, 2005)

Different studies suggest that assimilation of cholesterol is done by some microbes during their growth phase. Some strains of LAB like *Lactobacillus, Streptococcus, Enterococcus* and some pathogenic & nonpathogenic strains of *Mycobacteria* use cholesterol as a carbon source. Through different studies it is concluded that all above mechanism may work together to decrease the serum cholesterol level. Some of the mechanisms are found to be strain dependent(Kumar et al., 2012).

4.5.1 Cholesterol Assimilation by live cells:

The mechanism proposed by probiotics to remove cholesterol is cholesterol assimilation by live cells. It basically reduces the adsorption of cholesterol in the intestine. Probiotics must be in viable and growing phase in order to reduce cholesterol. In a study Tahri et al, (1995) stated that *Bifidobacterium spp*.in a growing phase assimilate cholesterol from the media containing bile salts. These is also another study which stated that assimilation of cholesterol by probiotics is not only done in aqueous media but also in semi-solid (butter, ice cream) (H Lye et al., 2010) . one thing observed is that presence of bile salt is related to cholesterol assimilation (Liong & Shah, 2005) but there is no relationship between the cholesterol uptake and nature of amino acid (li et al, 2013).

4.5.2 Attachment to the cellular surfaces:

There is another mechanism in which cholesterol level can be decrease through the binding to the cellular surfaces (Nguyen et al., 2007). It was considered when live cells with dead cells were able to decrease cholesterol level (Anandharaj et al, 2014), although the rate was very slow as compared to live cells. In a study it is found that exopolysaccharides production by probiotics are responsible for the adsorption of cholesterol (Tahri et al, 1995). (Ooi & Liong, 2010). In another study by Kimoto et al found that heat killed strains were also able to remove cholesterol from media but their ability was less as compared to live cells. This study has given the strength to the hypothesis that probiotics incorporate cholesterol in their cellular membranes. In another study by Honoso and Usman showed that *Lactobacillus bulgaricus* were able to attach cholesterol to their membranes.

Scanning electron micrograph of *L. bulgaricus* has given the clear results that the strains cultivated in media have cholesterol on their outer membranes as compare to media control.

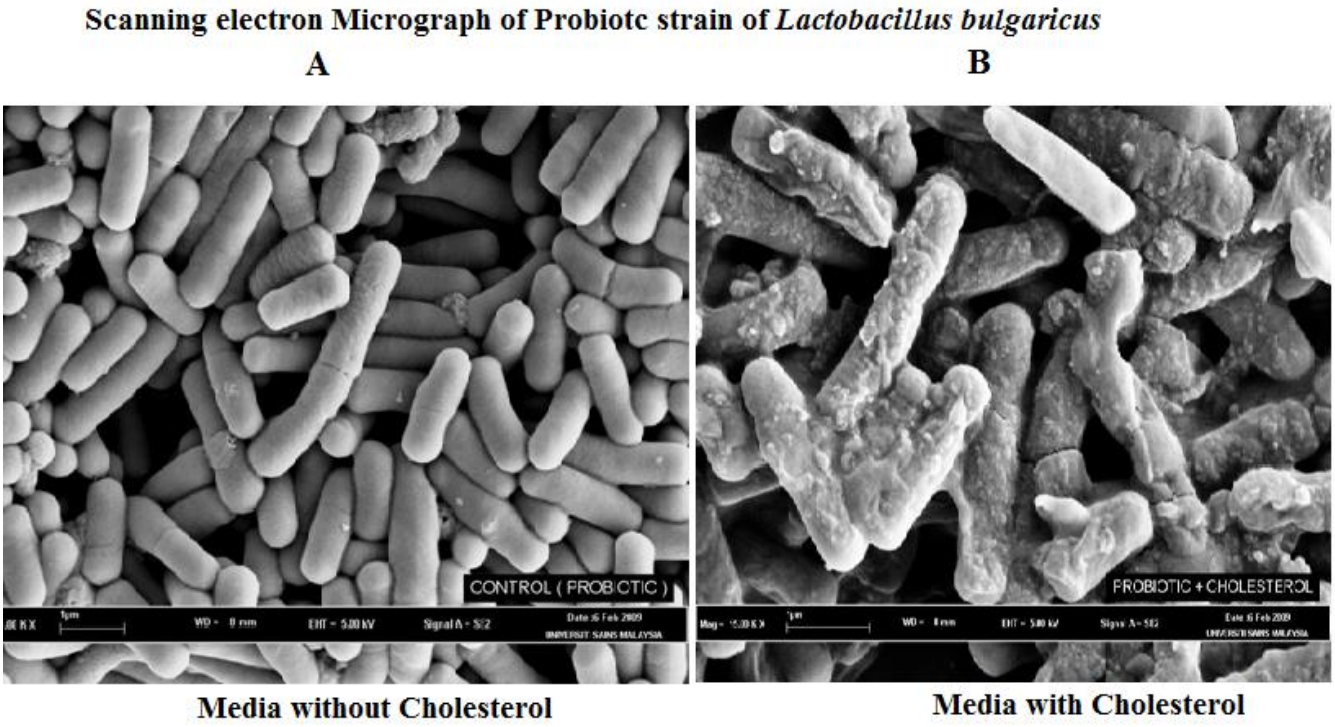


Figure 4: Cholesterol attachment to cellular membranes *(Ooi & Liong, 2010).*

4.5.3 Conversion into Coprostanol:

The third and the most interesting mechanism is conversion into Coprostanol, which is excreted through feces. In a study it is reported that all conversion in this mechanism is through different enzymes. First one is cholesterol dehydrogenase isomerase. There is one intermediate co-factor which is Cholest-4-en-3-one and is ultimately converted to Coprostanol by cholesterol reductase (H Lye et al., 2010). In vitro study cholesterol conversion by different *Lactobacillus* strains i.e. *L acidophilus, L. bulgaricus, L. casei* were assessed through fluorometric techniques. Increase concentration of Coprostanol due to fermentation process was also observed by removal of cholesterol in the media (Kumar et al., 2012).

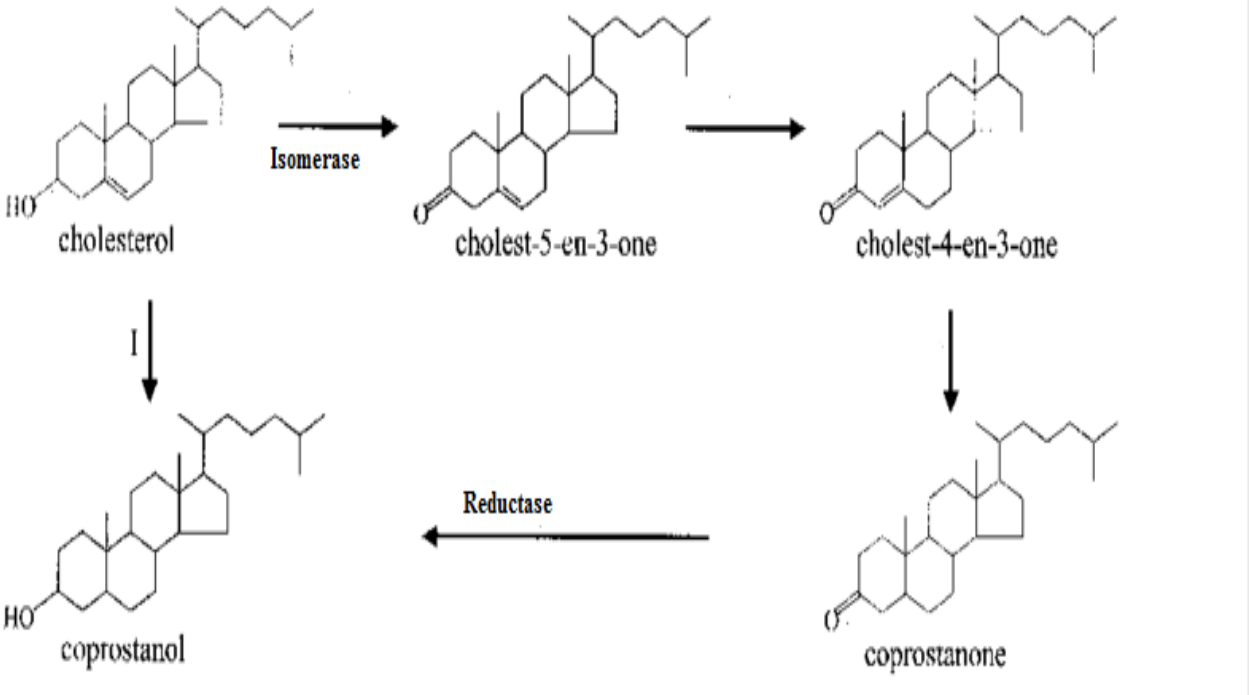


Figure 5: Conversion of Cholesterol into Coprostanol Adapted from (H Lye et al., 2010).

4.5.4 Incorporation into cellular membranes:

Many studies have reported that cholesterol reduction is also done by its incorporation into cellular membranes during growth phase (H Lye et al., 2010). In this incorporated cholesterol is unavailable and is not released for circulation. This results in decrease cholesterol level in humans. It has also been concluded that incorporation of cholesterol enhances the strength of the membranes and allows their growth and viability without the cell wall protection. In the studies it was found that cells of *L. acidophilus, Micrococcus lysodeikticus, Bacillus megaterium* and *Proteus mirabilis* showed some resistance when grown in bile salt media because of cholesterol incorporation into cellular membranes (lye et al., 2010). In a study Kimoto et al (2002) have examined the cholesterol reduction by different strains of *Lactococci*. They observed that there is a difference in the distribution of fatty acids pattern in cells grown in cholesterol media and without cholesterol media. Incorporation of cholesterol altered the fatty acids composition by increasing the saturated and unsaturated fatty acids concentrations. This increased concentrations leading to higher resistance and strength against lysis (lye et al, 2010). They also assessed the mechanism by incorporation of florescence probes into the bilayer membranes of probiotics strains. They observed that there is cholesterol enrichment in upper

phospholipid, polar head and tails of strain that were grown in cholesterol media as compared to control without cholesterol. These enrichment indications leaded to the incorporation of cholesterol in the regions (Ooi & Liong, 2010).

4.5.5 Bile salt hydrolase (BSH) enzymatic deconjugation:

The most interesting and studied mechanism of cholesterol is bile salt deconjugation in the intestine (Lye et al, 2009). Cholesterol is precursor for bile, which is formed in liver and water soluble. Upon ingestion of food it is released from gall bladder to duodenum where it is stored and concentrated. It consists of conjugated bile acids i.e. glycol or tauro conjugates, phospholipids, bile pigments and electrolytes. Conjugated bile salts are involved in fat emulsification. They have steroid ring conjugated with taurine or glycine. Bile salts act as signaling molecules in the regulation of glucose and cholesterol homeostasis. They also help in the inhibition of pathogenic growth through their antimicrobial potential in the small intestine. On deconjugation they become hydrophobic and are not absorbed by small intestine and easily eliminated through feces from the body (Kumar et al., 2012). Upon interruption of enterohepatic circulation of bile salts, bile salts synthesis increased which results in more cholesterol synthesis in liver.

Bile salt hydrolase (choloylglycine hydrolase) is the main component of enzymatic deconjugation mechanism. BSH activity is linked with cholesterol lowering potential of probiotics and is a desired characteristic of probiotics. It has been purified and characterized from different bacteria (Hill & Gahan, 2006) it is intracellular and sensitive to oxygen, its optimum pH is 5-6.

According to the studies different bacterial genera have BSH activity. The bacteria with BSH activity include *Bifidobacterium, Clostridium, Bacteroides, Lactobacillus and Enterococcus* (Sedláčková et al, 2015). *Lactobacillus and Bifidobacterium* are commonly used as probiotics other three are human GIT inhabitants. Studies suggest that BSH activity of *Lactobacillus* is less than *Bifidobacterium* (Tanaka et al, 1999).

Other than cholesterol assimilation, BSH is involved in Detoxification of bile salts and play an important role in the survival and colonization during the production in the harsh environment intestine/GIT. So it is a desirable trait of probiotics and is linked with overall health benefits. Thus BSH is main criteria for the selection of probiotics strains to reduce cholesterol

from the blood serum while non-conjugating bacteria do nothave potential to lower cholesterol from media (Tahri et al,1997)

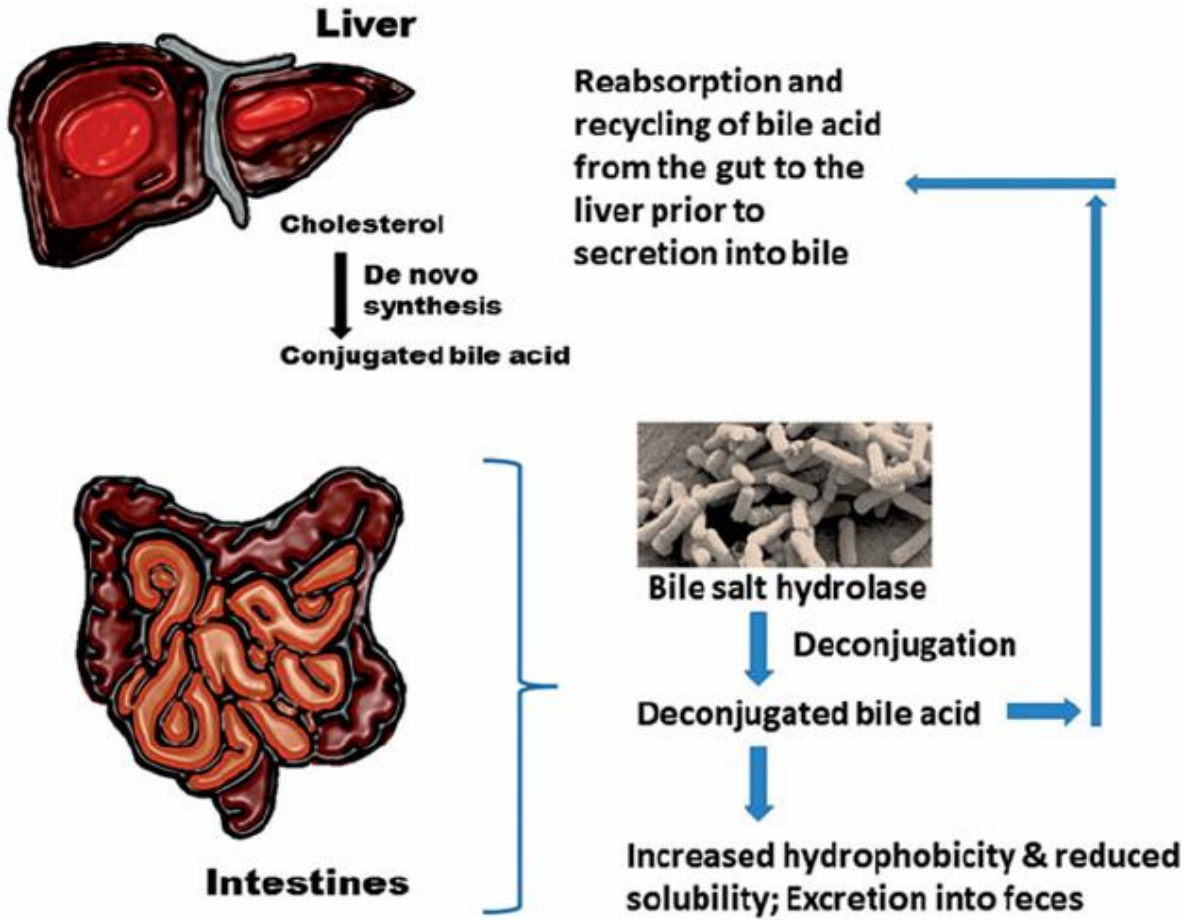


Figure 6 : Bile salt Hydrolase activity *Adapted from (Kumar et al., 2012).*

5. Materials and Methods

5.1 Reagents:

5.1.1 Phosphate Buffer saline:

The concentration used for PBS during experiment for serial dilution and washing was 1X. 20 tablets of PBS were added into 100ml of autoclaved water to make stock of 20X. This stock was stored at -20°C. $C_1V_1=C_2V_2$ equation was used to make desired concentration working solution.

5.1.2 Mac Farnald Standards:

It used to standardize the approximately bacterial numbers in a liquid by comparing the turbidity of Mac Farnald with the test suspension. Mac Farnald standard is mixture solution of Sulfuric acid and barium chloride, reaction of two results in barium sulfate (precipitation). The concentration of two standards is given below(Anonim, 2014):

Table 7: Composition of Mac Farnald standard:

Mac Farnald standard	Barium Chloride	Sulphuric acid	Cell Concentration/ml
0.5	0.05ml	9.95ml	2×10^8
5.0	0.5ml	9.5ml	1.5×10^9

5.1.3 Rifampicin Solution:

20 X stock solutions (20mg/ml) of antibiotic Rifampicin (sigma) was prepared by adding 500mg of antibiotics into 25ml of DMSO (Dimethyl sulfoxide). Mixture was filtered with 0.22µm syringe filter and stored at -20°C.

5.1.4 MRS Broth and Agar:

Media MRS broth and agar (sigma or Alpha) was used. 5.5 grams of broth and 7 grams of agar were added into 100ml of distilled water and autoclaved.

5.1.5 Diet composition:

For the induction of hypercholesterolemia, high fat contents were added into the normal feed. The diet ingredients were:

- Corn
- Wheat
- Soybean meal
- Wheat bran
- Dry milk powder
- Molasses
- Mineral mix
- Vegetable oil
- Antifungal agent
- Antioxidants
- Vitamins

Table 8: Composition of High fat diet & Normal Diet:

Composition	Percentage	**Percentage of Normal diet**
Protein	23%	23%
Fat content	14-15%	4-5%
Fiber	4%	4%
Calories	3700	2800-3000

5.2 In-Vitro Cholesterol Evaluation Test:

5.2.1 Exo-polysaccharide production:

All isolates were cultured in Exo-polysaccharide selection media (ESM) for 24 hours. Curd formation was observed. By vigorously shaking curd was broken and picked up by glass

rod / inoculation loop. Positive isolated were observed by ropy and long threads (lavanya et al., 2011).

5.2.2 Capsule staining method:

Isolates grown on skim milk media was spread on slide. It was then air dried and few drops of crystal violet were added on slides for two minutes. Then it was rinsed with 20% (w/v) $CuSO_4$ solution. Then it was observed under oil immersion 100 x lenses. Positive isolates showed capsule around their cell wall (Anthony, 1931)

5.2.3 Bile salt hydrolase Activity:

A qualitative method of BSH activity was performed; it was plate assay method for the screening of BSH Activity. For this MRS agar media was supplemented with 0.3% Bile salt (w/v) and 0.03% $Cacl_2$ (w/v). The pH of the media was adjusted to 5.5-5.7. Petri dishes were filled with MRS agar by puncturing at different spots. Different isolates were cultivated for 20-24 hours at 37°C under an anaerobic condition. These fresh bacterial strains were inoculated in the puncture agar and then incubated at 37°C for 72 hours under anaerobic atmosphere. Positive isolates formed halo zones or white precipitation around the puncture points. Bacterial strains grown on media without bile salt and $Cacl_2$ were taken as negative control (Sedláčková, Horáčková, Shi, Kosová, & Plocková, 2015).

5.2.4 Cholesterol Assimilation By live cells:

Cholesterol stock was prepared by adding 10ml of d.H_2O into 30 mg of cholesterol PEG-600, then filtered this with 0.22µm. 0.3% bile was added In the MRS broth. 1ml of stock cholesterol solution was added to the media then it was inoculated with 1% of active 24 hours probiotic culture and incubated at 37C for 24 hours under anaerobic atmosphere. Different time intervals of samplings were 6h, 12h, 18h & 24h. In this analysis bacterial cultures were centrifuge at 4°C for 20 minutes at 4000g. 2ml of ethanol (96%) and 1ml of KOH (33%) were mixed with the 1ml of the supernatant. After vortexing for 1 min, it was incubated at 37°C for 15 minutes. Then it was allowed to cool at 25-28°C. 3ml of hexane and 2ml of H_2O were mixed properly upon cooling. It was allowed to separate two different phases/layers. Upper layer was shifted to glass tube and residue solvent was evaporated under liquid nitrogen. 2ml of Ophthaldehyde solution was mixed with the dried residue followed by the vortex for the 1 minute. After mixing, 0.5ml of H_2SO_4 was added and allowed to cool at room temperature for 10

minutes after vortexing. Using UV spectrophotometer, the absorbance was read at 570nm (Tomaro-duchesneau et al., 2014),(Abd, Helim, Hashem, Essam, & Omar, 2016),

Cholesterol Assimilated (µg/ml) = Cholesterol (0h µg/ml) – Cholesterol (24h µg/ml)

Cholesterol assimilation % = Cholesterol Assimilated/ Cholesterol (0h µg/ml)

5.3 In-Vivo Safety and Survival Assessment of the potential probiotics strains:

5.3.1 Rifampicin tagging:

To locate the selected isolated in the field from the other micro-organism of the Animal. The isolated were tagged with rifampicin. For this antibiotic stress was given to the isolates by culturing in the different threshold of antibiotic (25µm/ml, 50µmg/ml, 100µg/ml and 200µg/ml). First of all, the isolates were grown in a MRS broth having initial concentration of rifampicin 25µmg/ml and incubated for 37°C for 20-24h under anaerobic conditions. Then MRS agar having same concentration of 25µg/ml of rifampicin were streaked by this broth and incubated for 48hrs at 37°C. Isolated colony picked from this media and inoculated in MRS broth of concentration 50µg/ml. Repeat these steps until the concentration of 200µg/ml of rifampicin. The tagged or resistant isolates colonies were again streaked on MRS agar with the concentration of 200µg/ml of rifampicin and evaluated for stability(Journal, 2014) .

5.3.2 Animal model:

Permission of rat models for experiment was obtained from ASAB internal board review. Approved form (IRB # 95) is attached. The 20 male Wister male rats aged 3 moths were taken from ASAB animal house and were divided into five groups of 4 each. Initially fecal samples of all the groups were collected and tested for rifampicin resistant by culturing them on agar plates containing 200µm/ml of rifampicin and incubate for 48 hours at 37°C under anaerobic atmosphere. All rats were preceded for the experiment having negative growth on plates. Each group was kept under standard condition i.e. 12h dark/12h light, 25-30°C, water availability for 24 hours and standard animal feed diet in a separate cage. The average feed was 35-40 grams per rat per day. And the water intake of rat was 25-30ml/rat/day. One group was taken as control while other four groups as experimental. Body weight and other measures were calculated properly during the trial.

5.3.3 Probiotic Dosage:

Each group was given a dosage of 2 x 10^8CFU/ml/day/rat. The dosage was administrated through drinking water to the rats. Normal drinking water was given to the control group. The overnight bacterial culture was centrifuge at 6000rpm for 10 minutes at 4°C. The supernatant was discarded and bacterial cells pellet was suspended in PBS and mixed properly. Wash the cell twice with PBS. After this Cells were mixed with drinking water and matched the turbidity of water with the Mac Farnald standard 0.5. Optical density of Drinking water with bacterial cells was noted after matching with the standard i.e. 0.08-0.1. Fresh dosage was prepared on the same day of administration.

5.3.4 Survival/Transit of isolates through GIT:

For the evaluation survival of potential probiotics through GIT that includes high acidic environment, enzymes, phenol stress and many gastric and bile juices. Fecal samples were collected for bacterial count at every 3rd day of feeding. Then 1gm of fecal from each group were collected and mixed in 9ml of PBS through vortexing. All samples were serially diluted up to 10^{-8} and all dilutions were plated on the MRS agar media of rifampicin concentration 200µg/ml. The spread plate method was used to calculate CFU/ml of the sample.

Colony forming Unit/ml = # of colonies X dilution Factor / Volume plated

5.3.5 Safety Evaluation of the potential probiotics isolates:

Rats were euthanized by using xylene and ketamine combination. Blood samples were collected from the pumping heart and immediately mixed in PBS solution before clotting. Liver, spleen, Large Intestine and small Intestine were harvested and placed in PBS solution. All organs and blood were mixed in PBS in through vortexing/ by sterilized pestle and mortar (Delgado & Rodrı, 2007). After homogenized mixing, all were serially diluted and were plated on MRS agar plates with 200µg/ml rifampicin and incubate for 72 hours at 37°C under anaerobic conditions to assess the viable bacterial count.

5.4 Evaluation of potential isolates on plasma cholesterol:

5.4.1 Animal Model:

22 male Wister rats of aged 8-12 weeks were divided into 5 groups. These rats were divided in 5 each for three experimental groups and three each for negative and positive control. All groups were given standard condition 12h light/12 dark condition, 25-28°C temperature, 24h

water availability and High fat Diet. The feed consumed by a rat was 30-35grams/day and water intake was 40-45 ml/day. Body weight was measured after and before the trail. All experiments protocols were permitted by Internal board Review. Copy of IRB form is attached.

5.4.2 Probiotic Dosage:

Each group was given the dosage of potential probiotic isolates of 1.5 x 10^9cfu/ml/day/rat. For this isolates were grown in MRS broth for 18-24 hours to reach the cell concentration of 10^9 cfu/ml. These isolates were pelleted by centrifugation at 6000 rpm for 10 minutes. The pellet was washed twice and again suspended in PBS. After this cell were mixed in drinking water of rats and matched their O.D with Mac Farnald standard 5.0. Negative control was given with plain drinking water while positive control was given statins (atorvastatin) 1mg/rat/day in drinking water.

5.4.3 Feeding:

Rats were fed with the normal diet for a week as in table 8. After this duration of adaptation, Negative control fed with only High fat diet (HFD)composition discuss in table 8 , Positive control fed with HFD and statins (1mg/rat/day). Experimental Group 1 fed with HFD and isolates I-2, Group 2 with HFD and isolate I-12, and Group 3 with HFD and isolate I-17. All groups were fed for 30 days. All activity, behavior, food and water intake of rats were monitored daily (Abd et al., 2016) (Singh, Malik, & Katkamwar, 2015).

5.4.4 Cholesterol Analysis:

Rats were euthanized with ketamine and xylene combination with help of veterinarian.0.5ml of Blood sample was collected from the pumping heart into sterile tube (for complete lipid profile) at 0 day and 30^{th} day from each rat followed by food deprivation for 8-10 hours. Heart liver, Kidneys and spleen were qualitative analyzed. Blood samples were taken to the ASAB Diagnostic Lab(NUST) (Abd et al., 2016)

6. Results

6.1 Isolate history:

Four potential probiotic strains were selected that were previously isolated by lab fellow (A Muneera, (2017). They were already evaluated for their cholesterol reducing ability. They had maximum cholesterol reduction ability than all other isolated strains. Re-evaluation of these potential probiotic strains were conducted in this study as shown in table 9.

Table 9: Source of selected probiotic strains:

Strain #	Origin	Strain name	*In vitro* Cholesterol Assimilation
I-2	Milk	*Lactobacillus rhamnosus*	37%
I-12	Milk	*Lactobacillus rhamnosus*	32%
I-7a	Milk	*Lactobacillus salvarius*	16%
i-17	Pickle	Unidentified	52%

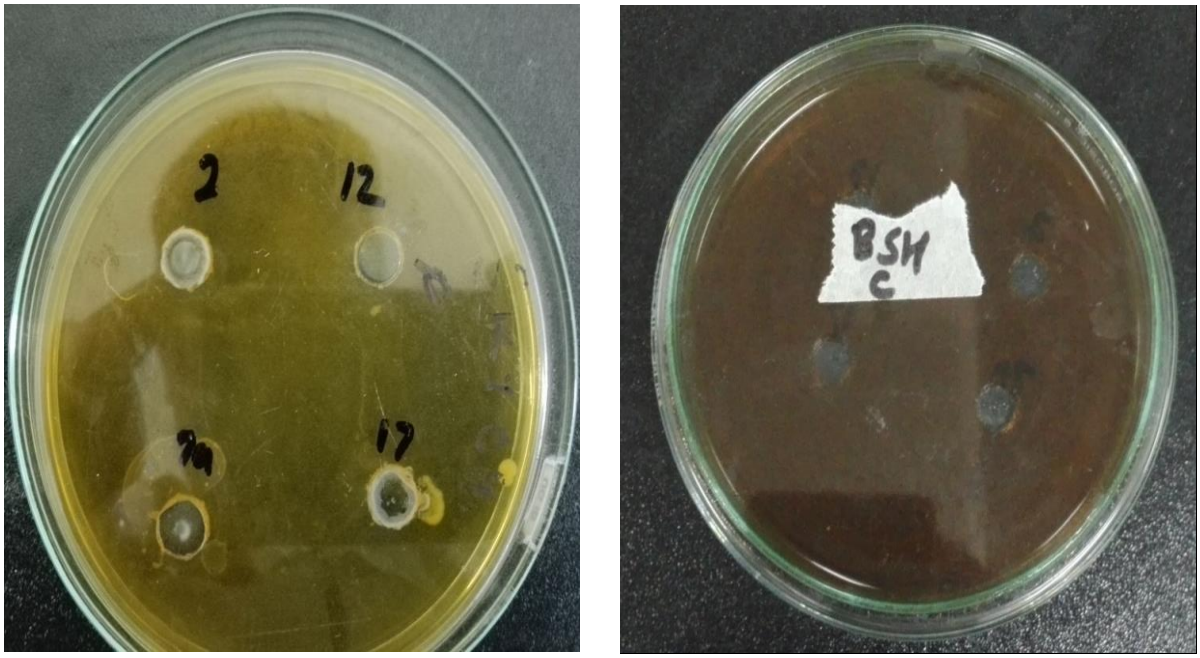
6.2 Exopolysaccharides production:

6.2.1 Assessment of EPS probiotics strains:

Although the isolates appeared to have slimy colonies on MRS supplemented with L cysteine, however no apparent ropy strands were formed. The similar was observed in curd formed by skim milk by individual isolates. These findings were supported by negative results after copper sulphate staining.

6.3 Bile salt hydrolase activity:

Qualitative plate assay method was used to evaluate BSH activity. White precipitation or hollow zones were considered as positive BSH activity. The white precipitation formation around the strain I-2 and I-17 i.e. *Lactobacillus rhamnosus and Lactobacillus (I-17)* species had shown the BSH positive activity while others two strains *L.rhamnosus and L.salvarius* had slight or no BSH activity as shown in fig8.



A **B**

Figure 7: Bile Salt Hydrolase Qualitative plate assay

(A) Media supplemented with Bile salts. Formation white precipitation around I-2 and I-17 showed positive BSH activity while others showed no significant activity. (B) Media without bile salts as control, No white precipitation were observed due to no enzyme production.

6.4 In-vitro Cholesterol Assimilation of the potential probiotics isolates:

All isolates were evaluated for in vitro cholesterol assimilation. Variable cholesterol reduction ability was observed by all these selected probiotic isolates. The cholesterol assimilation by these potential isolates was between 19 to 48% as shown in fig 9. The maximum activity was observed by *L. plantarum ATCC 14917* i.e. 48 % while the minimum cholesterol reduction was assessed by *L. salvarius i.e.* 19%. The cholesterol assimilation by other two strains of *Lactobacillus rhamnosus* (I-2 & I-12) was approximately 30%. The experimental strain I-17 i.e. *Lactobacillus* species had maximum cholesterol assimilation i.e. 42% comparable with the reference strain of *L. plantarum ATCC 14917*. *L.plantarum ATCC 14917* strain was used as positive control.

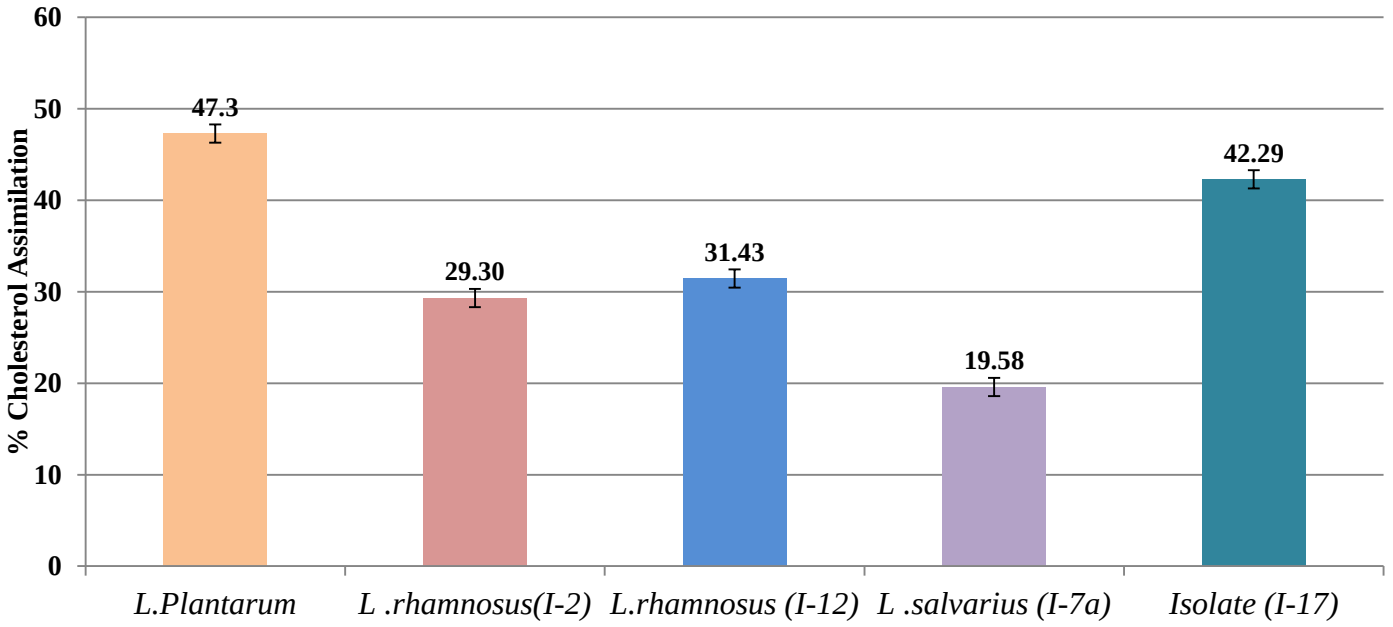


Figure 8: Comparison of Cholesterol Assimilation by *Lactobacillus* strains.

Error bar represents the standard deviation from the mean (n=3)

6.5 In-vivo Survival of the potential strains:

. Their survival and colonization of the selected strains were evaluated in rat model. The post mortem examination of test animals revealed no apparent signatures of infection, in addition no mortality during the experimental phase of 28 days was observed in animals under trail.

6.5.1 Survival through the GIT:

The selected potential probiotic strains survived the GIT transit and which is evident from the retrieval of these isolates from rat feces. This observation was showing the viability and survival of strains through the GIT indicating that potential strains were not only able to survive but also colonize the intestinal walls. No colonies were obtained in the control group during the whole trial which shows no cross contamination of isolates among various test groups

Bacterial cell count in the fecal samples was increased during the 12^{th} day of trial and then it started stabilizing which reflects that they were survival and colonization in GIT as in fig 10.

.

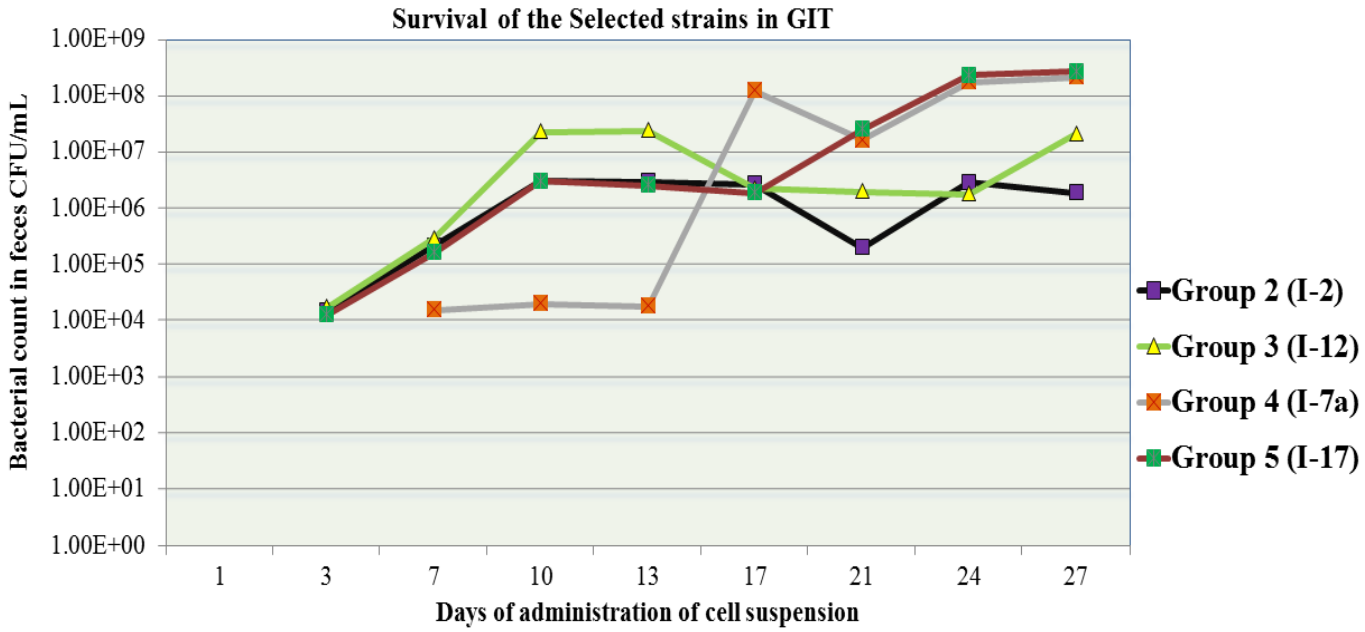


Figure 9: Recovery of tagged cells during the Study (0 day to 30th day)

Cells collected from the feces of the rats at different days. Dots on the line represent the standard deviation

6.5.2 In vivo safety & colonization of the strains:

The colonization preferences of the rifampicin tagged strains were evaluated in the large and small intestine collected from the animals. For the safety assessment Liver, spleen and blood were taken and homogenized. All the strains showed colonization in the large intestine as well as in the small intestine while better colonization was in the large intestine as comparison to the small intestine. Maximum colonization i.e. 7.81 x 10^7cfu/ml was shown by strain *L.rhamnosus* (I-12) in the large while in small intestine was 2.55 x 10^6cfu/ml**.** No colonization in the control group was observed (fig10). No rifampicin tagged colonies were observed from the blood, liver and spleen samples which reflect no pathogenicity and safe for other trails.

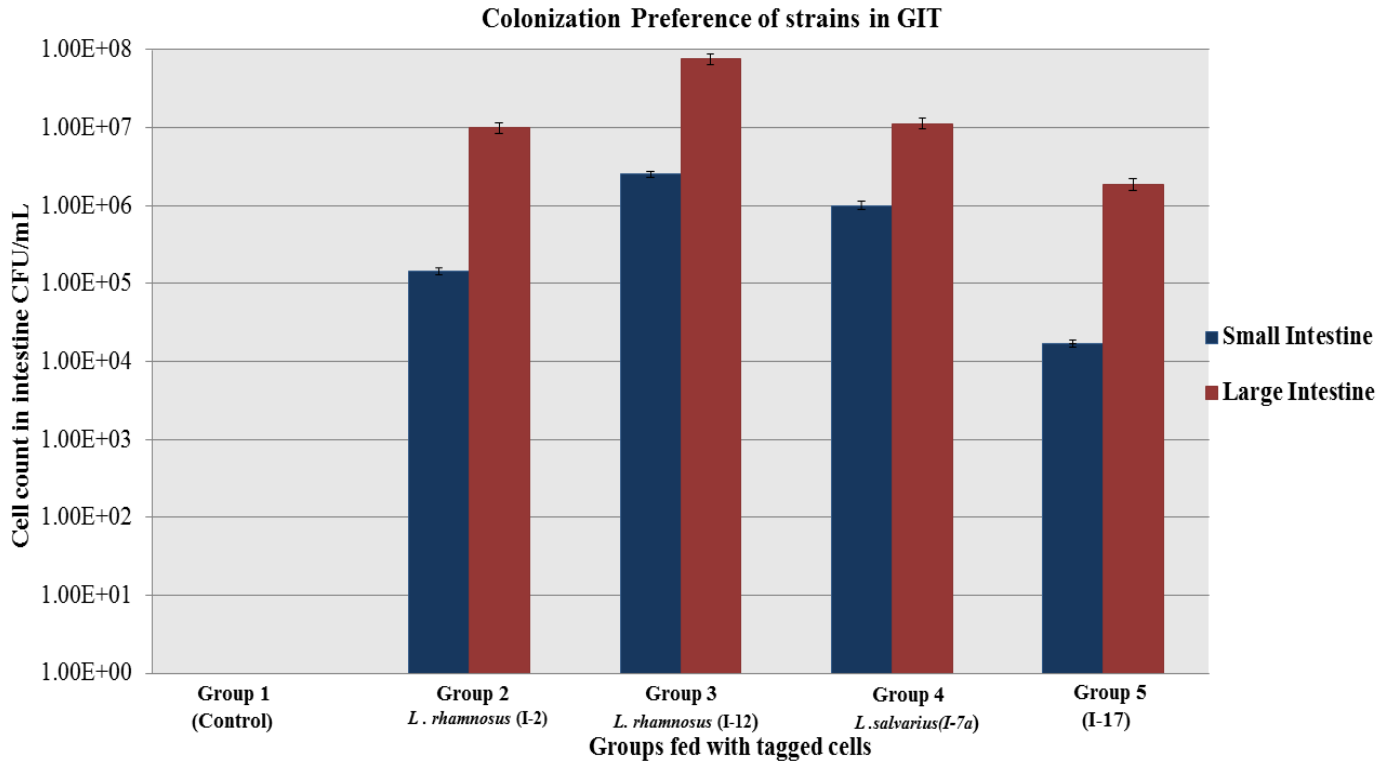


Figure 10: Recovery of Strains from Small & Large Intestine in 30 days

Error bar represents the standard deviation from the mean (n =2 rats)

6.5.3 Impact of isolates on the average weight gain of the rats:

Weight gain is used as a standard for rat health. The weight gain was observed after 28 days of trail in all test animals used in this study. However the extent of weight gain varied across different groups. More weight gain was observed in control group that is approximately 27% while in all other experimental group weight gain was between 12 to 16 % as indicated in fig 11.

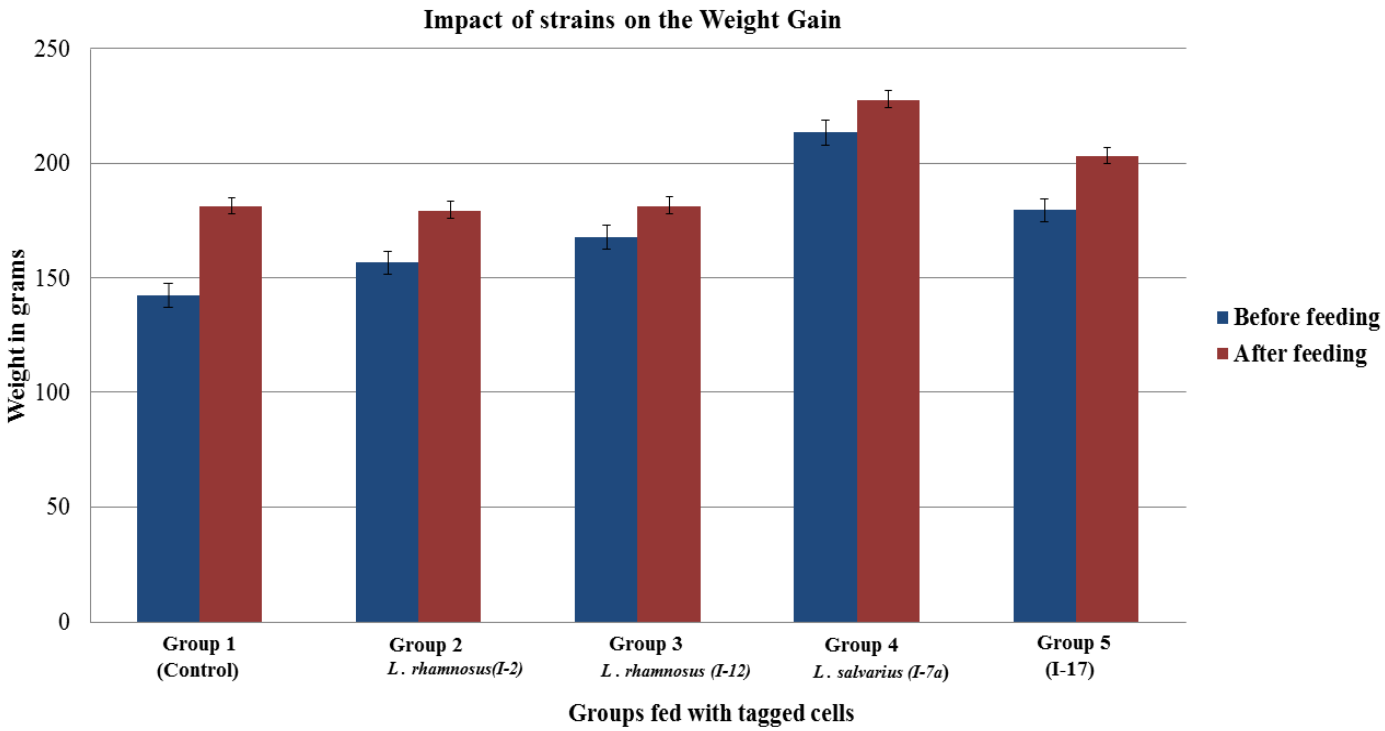


Figure 11: Weight of rats during the trial

Error bar represents the standard deviation from the mean (n=4 rats)

6.6 In vivo cholesterol reduction Assay:

On the basis of in vitro results, three potential probiotic strains were selected and evaluated for in vivo cholesterol reduction ability. 8% Increase in cholesterol concentration was observed in control group as well as increase in LDL and decrease in HDL ratio. Variable cholesterol reduction was observed by the entire experimental group but the maximum cholesterol reduction was shown by *L. rhamnosus (I-12*) i.e. 26% while the less performing isolate *L.rhamnosus (I-12)* showed the comparable results with the best performing drugs statin i.e. 15%. The other *Lactobacillus (I-17)* group had 24 % cholesterol reduction. In case of LDL and HDL, the increase in HDL was between one to two folds while decrease in LDL was 2 to 3 fold, The best ratio was by *L.rhamnosus (I-12)* that was three fold decrease in LDL and one fold increase in HDL level as in fig 12. Statin which is globally used drugs for cholesterol reduction used as positive control. However no reduction in triglycerides level was observed by all the isolates except I-17 which showed no change in triglycerides level.

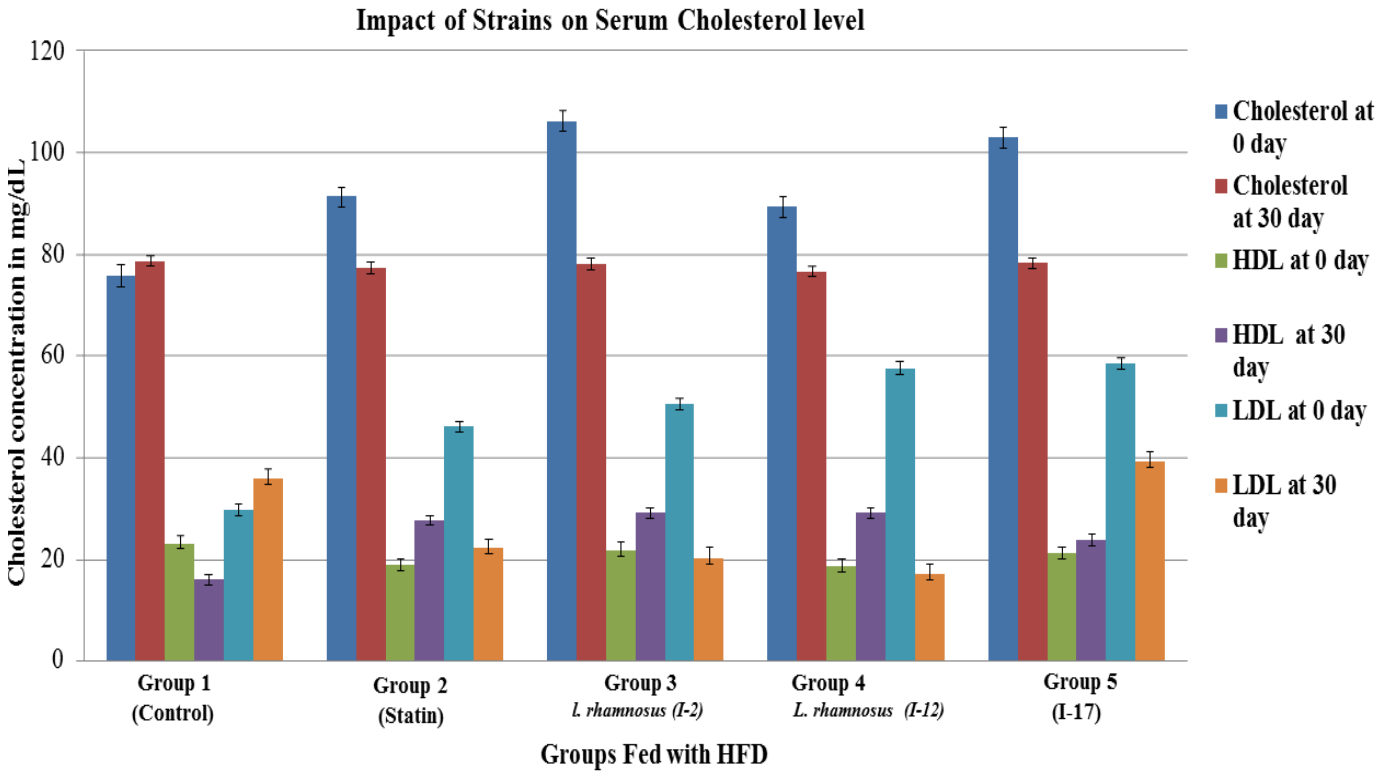


Figure 12: *In vivo* Serum Cholesterol Reduction

Error bar represents the standard deviation from the mean (n=5 rats)

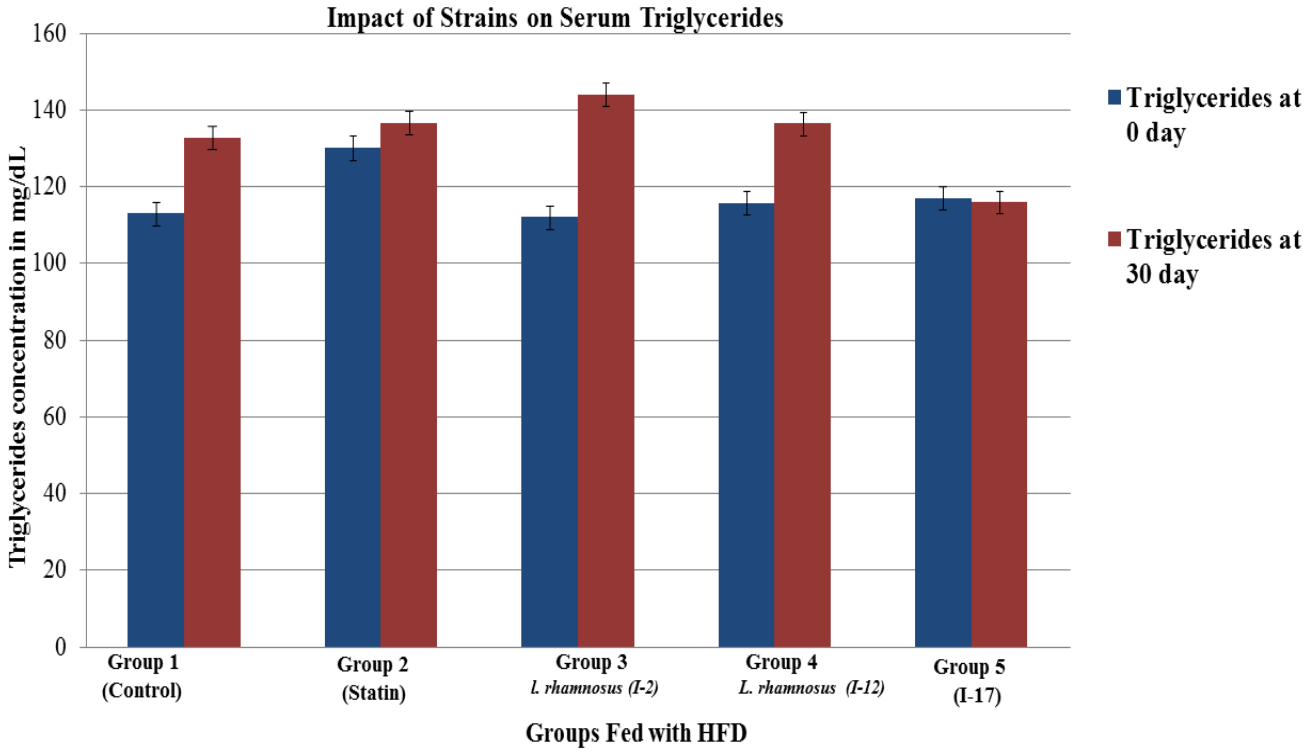


Figure 13: In-vivo Triglycerides Evaluation

Error bar represents the standard deviation from the mean (n=5 rats)

Table 10 Impact of potential probiotic on plasma cholesterol:

Groups	Cholesterol	HDL	LDL	Triglycerides
Control	4% Increase	30.43% D	20.68% I	17.39% I
Statin	15.34% Decrease	48.23% I	50.17% D	4.61% I
I-2	26.50% D	34.25% I	60.31% D	28.50% I
I-12	14.31% D	49.46% I	49.46% D	17.70% I
I-17	24.80% D	12.38% I	32.64% D	0.34% I

6.6.1 Impact of potential probiotic strain with HFD on the weight gain of rats:

Weight of the rats was measured before and after the trial. Variable weight gain was observed all the groups. The weight gain in control and statins group was up to 160 grams while all other probiotic isolates groups weight gain was between 100 to 120 grams (fig 14) because probiotics help in the digestion and some strains also use in weight lost. This means that these strains can be used as weight watcher after further analysis.

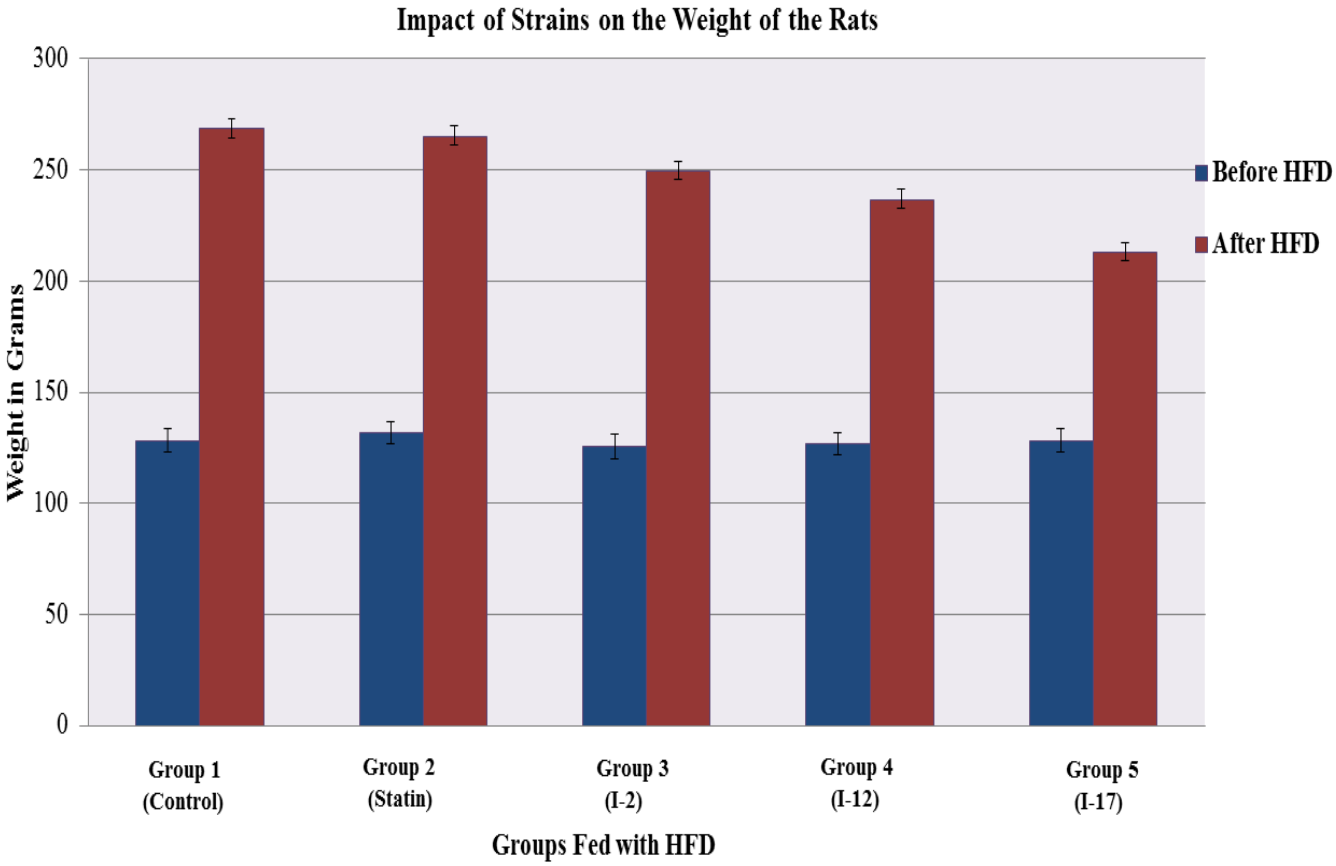


Figure 14: Ability of strains on Average weight gain

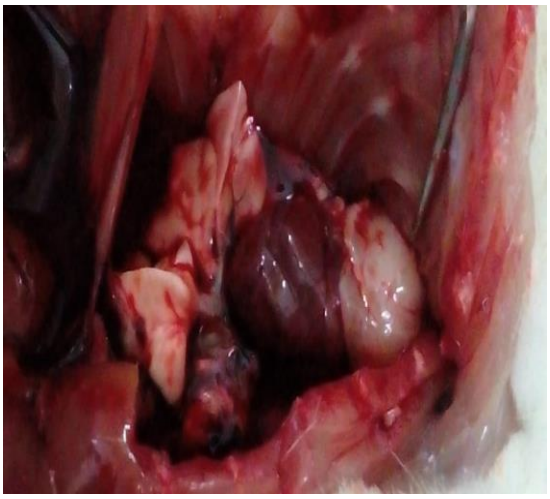
Error bar represents the standard deviation from the mean (n=5 rats)

6.6.2 Anatomical examination of rats' organs for the fat content:

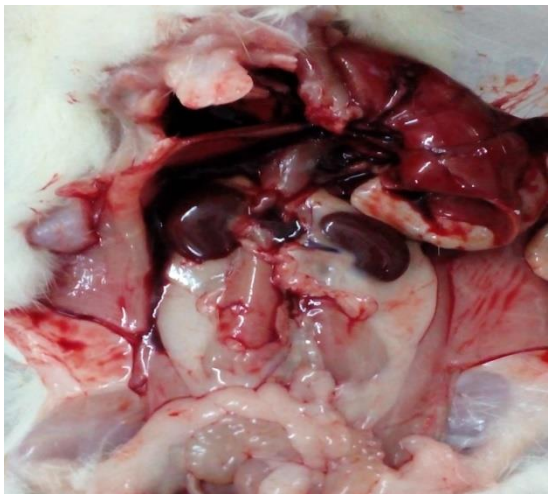
Rats' organs were qualitative analyzed for the fat content. Fatty layers around the heart liver and abdomen were observed in control group (fig 15). The alarming condition is the fatty

layers around the heart which can be the cause of different heart diseases. There was no fat projection or fatty layers in all the potential probiotic groups and even in Statin group animals. Potential probiotic isolated group have weight gain but no fat growth on any rat organ suggesting that these isolates have potential to reduce cholesterol and no effect on overall health.

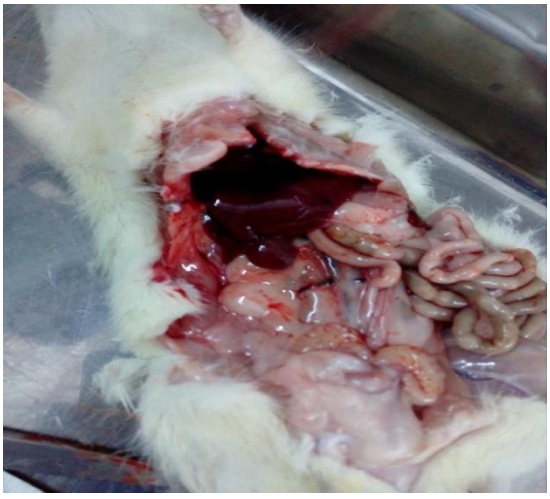
Anatomical Analysis of Rats



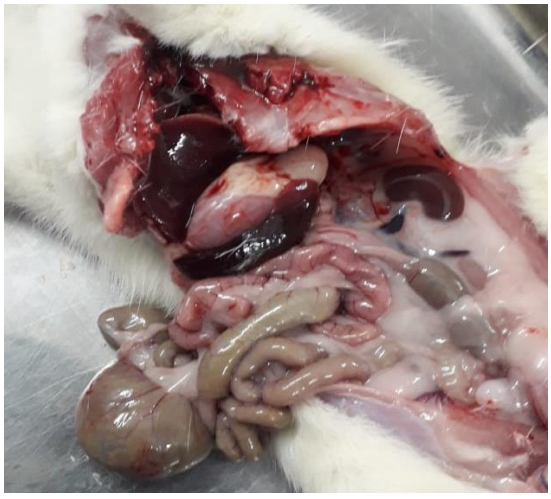
A 1`



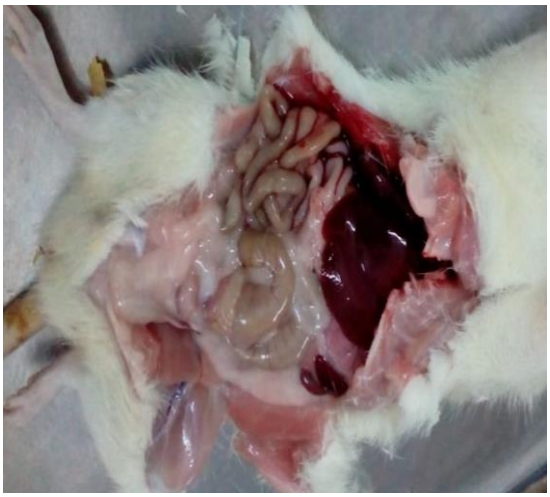
A 2



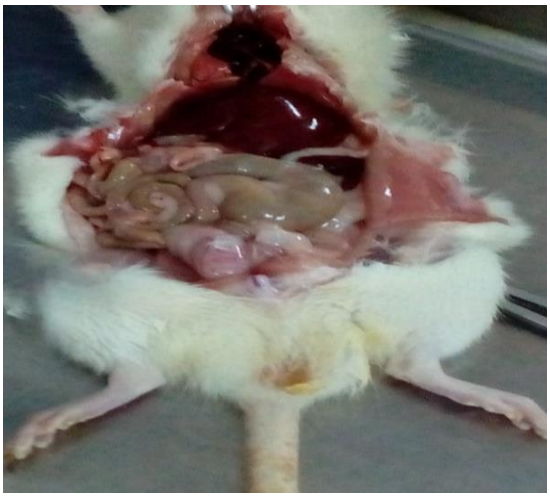
B 1



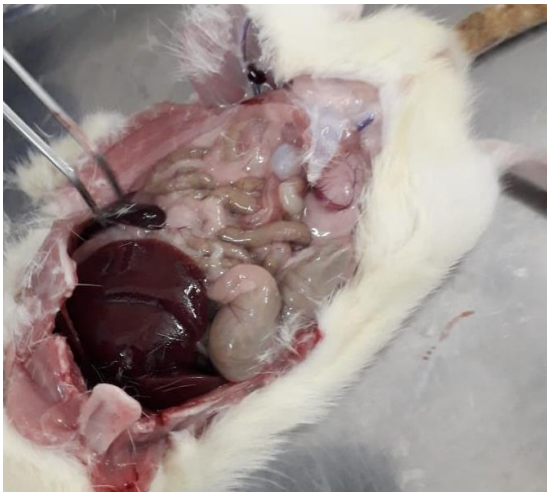
B 2



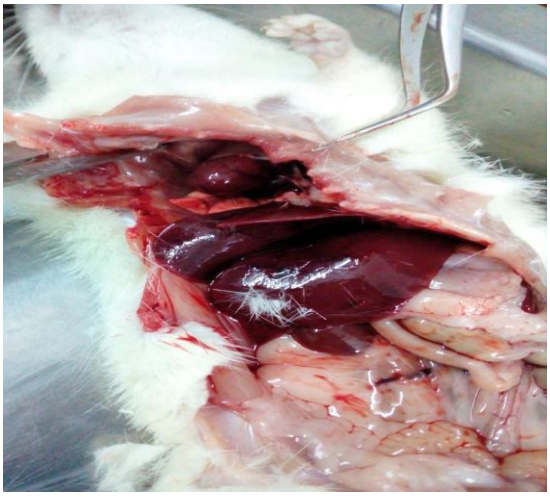
C 1



C 2



D 1



D 2

Figure 15: Anatomical Examination of rats' organs

[A (Control)] All of the organs have fatty layers and fat projection around them [B (Statin)] Less fatty layers around any of the organs [C (I-2) & D(I-17)] No fatty layers around any of the organs

7. Discussion

In this study, previously isolated four *Lactobacillus strains* were selected on the basis of their potential. These four have not commercial usage history so it is necessary to assess their safety and survival profile in GIT. In the first phase of study we assessed their survival and safety in rat model. All of four strains showed their survival and safety in the rat model. These probiotics have different potential benefits like maintenance of gut micro-biota, liver diseases and cholesterol reduction. CVDs are the main cause of deaths these days and high blood cholesterol is highly associated with CVDs. Different probiotics strains have already been used to treat high cholesterol level. In this study we evaluated the *Lactobacillus* strains for their cholesterol reducing ability in vitro and in vivo. *L.rhamnosus (I-2) and Lactobacillus* (I-17) strain significantly reduced the total cholesterol level and also increase the HDL/LDL level. Both of these also showed BSH positive activity and also had better results in cholesterol assimilation test in broth medium.

7.1 Safety, Survival and Colonization in GIT of rat:

For an ideal candidate the probiotic strain should be bile, acid and phenol tolerant and also they should have ability to colonize to GIT. For this selected strains were evaluated on rat model because rat model is similar to real conditions and give significant and trust worthy results. Rats are like humans in mono-gastric terms but there is a lot of anatomical differences between these two (Tannock, 1999). The results of trial stated that these strains were survived in the GIT and easily compete with micro-biota of the host. These strains were obtained from the fecal samples after 2nd day dosage. These strains showed the survival and also had no symptoms of infection.

To locate the given strain from the indigenous micro-flora of rat, these strains were rifampicin tagged. This rifampicin tagging remains for approximately 20 generations and is non-transferable. Due to these, it is an efficient protocol to locate tagged bacteria from the others in the field. According to the Frece et al (2005) and Fujiwara et al (2001), Antibiotic tagging is the most competent method to locate desirable bacteria from the other. These strains endured the conditions of GIT and were collected through fecal sample during the whole trial. During the first week, the count was low indicating the adaptation period in the GIT harsh environment. After this count increased and starting constant, showing their colonization and adhesion to the

GIT. In a study by Frece et al (2005) , same protocol was adopted , In this study three rifampicin tagged strains were tested for survival and safety profile in a mice model.

For the colonization of these strains the small intestine and large intestine was taken. The colonization preference was different for different strains because its strain specific property. It can easily state from results all of our strains have preferred large intestine over small intestine, although small intestines have colonization but large intestine have colonization preference. Maximum colonization in the large intestine as well as in the Small intestine was shown *by Lactobacillus rhamnosus (I-2).* Mostly *Lactobacillus* strains colonization preference in large intestine and these strains also have this property. In a study in which probiotics strains showed preference as these strains shown for large intestine, which comply my study as all of four strains showed preference towards large intestine (Frece et al, 2005). According to different studies *Lactobacillus* species have adherence preference towards large intestine and there are some strains of L.fermentum which have preference towards spleen and small intestine (Ks et al, 2013)(De Champs et al, 2003). Another study showed that *L.rhamnosus SKG34* also have potential to colonize in the vaginal tract but also colonization preference towards large intestine (Uni et al, 2013).

In this study rats were fed with probiotic cells for 4 weeks to evaluate the survival and safety of these isolated strains of *Lactobacillus*. During the trial weight gain of rats were observed. After 4 weeks rats were sacrificed and liver, spleen and blood were taken to assess their safety and pathogenicity. No bacterial isolates were found in liver, spleen and blood. This showed that these are safe for human and pharmaceutical consumption. In many studies weight gain has also been shown an indicator of good health. These safety results are same with study of Bhardwaj et al (2010), in which some potential probiotics are safe for human usage with the same no growth in any organ stated they are safe for human usage.

In another study they evaluated the safety of *Lactobacillus* strains, no bacterial growth were observed in liver, spleen and blood showing that they had no pathogenicity and safe for human usage. Our results are congruent to the studies which comply and strengthen our findings. Different studies by Saarela et al (2000) stated that safety is the main selection criteria for any probiotic strains and there has been a debate about safety testing of new strains for human consumption. One of the proposed criteria is the absence of infection and pathogenicity is taken

as important factor for safety consideration (Huang et al, 2003). In our study these four strains have same results with no pathogenicity and infection in the rats. And also they had no effects on food & water intake, general health status and weight gain. And also there was no bacterial translocation in any organ or even in blood.

7.2 *In vitro* Cholesterol Assimilation Assay:

In the second phase of study, in vitro cholesterol assimilation was evaluated by both qualitative and quantitative methods. In first qualitative assay, two strains I-12 and I-17 i.e. *Lactobacillus* rhamnosus and unidentified strains was BSH positive results, this mean that these both strains have ability to reduce the cholesterol. These both strains produce enzyme bile salt hydrolase enzyme, which is key factor for cholesterol reduction. This enzyme is main component of bile deconjugation mechanism of cholesterol reduction. But two out four showed significant BSH positive results while other didn't show significant activity because their isolation source is dairy products and no animal gut or fecal sources. It has been observed by many studies that habitat of strains plays main role in the BSH activity presence ((Sedláčková et al, 2015). GIT and Bile stress environments are likely to have BSH significant activity because they have ability to survive the stress environment. They resist bile through bile salts deconjugation mechanism and deconjugating bile intro free bile ((Kumar et al., 2012). While there is no bile stress to the bacterial habitat of this environment, so these isolated sources strains have less or low ability of BSH (Begley et al, 2006).

This was only a qualitative test and probiotics have five proposed mechanism, so these four strains were further evaluated for in vitro cholesterol reduction ability. The cholesterol assimilation ability was between 19 to 43%. These results are supported by different studies because different studies stated that *Lactobacillus* strains have ability to lower the cholesterol between 15 to 55 %. Strains of *Bifidobacterium* have more cholesterol lowering ability than *Lactobacillus strains* (Abd, Helim, Hashem, Essam, & Omar, 2016) The results of this study strengthen our findings about the cholesterol lowering ability. The maximum cholesterol reduction was approximately 43% and shown by I-17 i.e. *Lactobacillus* (unidentified) strain and minimum was 19 and by I-7a i.e. *Lactobacillus salvarius* while other *two L.rhamnosus* strains have 30% cholesterol assimilation results. The main reason that I-17 and I-2 better assimilation was that they have also significant BSH activity while others have not significant activity. This also strengthens the existing hypothesis that BSH activity is the main role in reducing the

cholesterol blood level (Kumar et al, 2012). These findings are also in line that observed by Uni et al (2012) who reported in vitro studies of two strains *Lactobacillus rhamnosus SKG34 and FBB42* have ability to reduce the cholesterol level. In a study by Tomaro et al, (2014) reported that when *L.rhamnosus* were used in high dosage, this was able to assimilate 30% of the cholesterol in vitro conditions. Our findings have the same results that both of *L.rhamnosus* strains were able to assimilate approx. 30% of cholesterol and this comply our findings with the same results.

Weight gain was observed by all the isolated but the gain was less as compared to control because *lactobacillus spp* have the ability to work as weight watcher. Many studies showed that some of the strains have the ability to control the weight and help in weight loss. Some of the commercial products of probiotics are available in market which used to reduce the weight.

In vitro results of *Lactobacillus* strains for cholesterol reduction has been used as a selection tool of potential strains with different health characteristics (Chávez et al, 2015). The in vivo ability of probiotic strains could be proportional to cholesterol reduction capability in medium enriched with cholesterol (Gilliland et al, 2008). According to their studies, only 2µg/ml in cholesterol reduction by different *Lactobacillus* strains resulted in different cholesterol reducing lipid concentrations. Cholesterol assimilation from the media was due to different mechanism of probiotics so further in vivo assessed is necessary for their cholesterol reduction ability.

On the basis of in vitro cholesterol assimilation, three strains were selected and further were evaluated their ability in rat models. In this trail all of the groups were fed with high fat diet because high fat diet is used for increasing cholesterol level in blood serum. The ability of rats was compared with the conventional statin medicine (atorvastatin) which is mainly prescribed by doctors for cholesterol reduction. The results of this trial showed significant cholesterol reduction as well as reduction in LDL level. This also stated that these strains have capability of increasing in HDL level. All of strains showed cholesterol reduction but maximum cholesterol reduction was done by I-12 and I-17 i.e. *L.rhamnosus and Lactobacillus* (unidentified). The cholesterol reduction by statin group was 15 % while I-2 and I-17 had 25 % cholesterol reduction ability. This means that these strains have better ability than statins. And in case of LDL level, statins reduce the LDL level by one fold while both L.rhamnosus strain showed 2 and 3 fold reduction of LDL concentration. While HDL was increased in all strains and statin group in the

same concentration. In case of Triglycerides, all were able to control this at a static manner but I-17 was also able reduce this. In overall blood lipid profile comparison I-17 was shown the same ability as statin had. But statins have many adverse effects while probiotics have many other health promising benefits and no side effects. While in cholesterol reduction *L.rhamnosus* was able to give better result than statins. The least performing group has the comparable results with the statins.

7.3 Impact of potential strains on plasma cholesterol:

According to in vivo studies, *L.rhamnosus* has ability of cholesterol reduction by 22%. This study strengthens our findings and stated that these strain can be used to reduce cholesterol level (Ooi & Liong, 2010). Different studies stated that LDL is the key component of cholesterol serum. Therefore LDL reduction is an important factor for cholesterol reduction. All of our three strains were able to reduce the high LDL level in rat model caused by HFD. Study by Park et al(2007) reported that *Lactobacillus* strains have the ability to reduce the LDL concentration in animal model. This complies with our findings. Similar results were shown by Uni et al and Ha et al (2006) that Two *Lactobacillus* rhamnosus strains SKG34 & FBB42 appeared to reduce cholesterol level in rat model fed with HFD. Our study stated that *Lactobacillus* strain played the key role in the risk reduction of cardiovascular diseases. The maximum cholesterol reduction was shown by I-2 and I-17, these both strains was also BSH positive. On the basis of these findings we can state that BSH is the key component for cholesterol reduction ability. This point is also strengthening by a study by Lee and Salminen (2006). In this study they reported that *Lactobacillus* strains with Bile salt hydrolase activity have better cholesterol reduction in in vitro and in vivo conditions. But the mechanism by which these strain reduce the cholesterol is unclear up till now.

8. Conclusion & future aspects

The current study showed that all four strains have potential probiotics characteristics. All of them are safe and have no sepsis, infection and pathogenicity symptoms in rats. In both of the *in vivo* trials weight gain was observed by all the potential probiotic fed rat groups which is considered as standard for the measurement of health of the rats. Variable weight gain was observed by the different groups. The weight gain in potential probiotics group was less than the control group so they may have the potential to work as a weight watcher. These strains have ability to colonize the GIT and ability to survive the harsh environment of GIT. In qualitative analysis for BSH activity, two strains i.e. I-2 and I-17 have BSH positive activity which is considered as a primary test for cholesterol assimilation activity. The maximum cholesterol reduction in broth medium was also observed by BSH positive strains i.e. I-2 & 17. In case of plasma cholesterol all of these have ability to lower the cholesterol serum level better than statins without any side effects in rat models. These potential probiotics have the ability to enhance the HDL & LDL ratio better than globally used drug statin. In the anatomical examination of the rats, no fatty layers were observed in the isolates group as compared to control, so these can be used as weight watcher.

In the future, these strains can be used in human trails for further analysis. Their Adhesion and Immuno-modulation can be evaluated on cell lines. However in future, there is need to evaluate more isolates with better ability. We can go for complete genome sequences for their further characterization. On the basis of genome we can predict their potential mechanism for cholesterol reduction. By evaluating other technological aspects these can be used as an alternative treatment for hypercholesterolemia. Assessment of cholesterol assimilation can be evaluated with synbiotics and isolates with better potential can be used for functional food product development.

9. Reference

Abd, M., Helim, E., Hashem, S., Essam, T., & Omar, M. (2016). Clinical Microbiology : Open Access Cholesterol Lowering Capability of some Lactobacillus Strains and its Effect on Mice Fed a High Cholesterol Diet, *5*(3), 3–6. https://doi.org/10.4172/2327-5073.1000253

AbuMweis, S. S., Barake, R., & Jones, P. J. H. (2008). Plant sterols/stanols as cholesterol lowering agents: A meta-analysis of randomized controlled trials. *Food and Nutrition Research*, *52*, 1–17. https://doi.org/10.3402/fnr.v52i0.1811

Al-awwad, N. J., Takruri, H. R., & Yamani, M. I. (2014). Effect of Probiotic Hummus on Blood Lipids of Rats, *7*(4), 261–267.

Anandharaj, M., Sivasankari, B., & Parveen Rani, R. (2014). Effects of Probiotics, Prebiotics, and Synbiotics on Hypercholesterolemia: A Review. *Chinese Journal of Biology*, *2014*, 1–7. https://doi.org/10.1155/2014/572754

Anonim. (2014). Mc Farland Standard. *DALYNN Biological*. https://doi.org/TM50-TM60

Anadón, Arturo & Martínez-Larrañaga, Maria & Ares, Irma & Aránzazu Martínez, María. (2016). Probiotics. 777-798. 10.1016/B978-0-12-802147-7.00055-3.

Begley, M.; Gahan, C. G. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Review*, vol no. 4, pp. 625-51

Badel S., Bernardi T., Michaud P. (2011). New perspectives for Lactobacilli exopolysaccharides. *Biotechnol Adv.* **29**: 54–66.

Bliznakov, E. G. (2002). Lipid-lowering drugs (statins), cholesterol, and coenzyme Q 10. The Baycol case–a modern Pandora's box. Biomedicine & pharmacotherapy, 56(1), 56-59.

Chávez-tapia, N. C., González-rodríguez, L., Méndez-sánchez, N., & Uribe, M. (2015). Current evidence on the use of probiotics in. *Journal of Functional Foods*, *17*, 137–151. https://doi.org/10.1016/j.jff.2015.05.009

Chen, Z., Ma, K. Y., Liang, Y., Peng, C., & Zuo, Y. (2011). Role and classification of cholesterol-lowering functional. *Journal of Functional Foods*, *3*(2), 61–69. https://doi.org/10.1016/j.jff.2011.02.003

Reference

Charbonneau, D. H., & Healy, A. M. (2005). Genetics Home Reference. *Journal of Consumer Health On the Internet*, *9*(4), 61–68. https://doi.org/10.1300/J381v09n04_07

Chávez-tapia, N. C., González-rodríguez, L., Méndez-sánchez, N., & Uribe, M. (2015). Current evidence on the use of probiotics in. *Journal of Functional Foods*, *17*, 137–151. https://doi.org/10.1016/j.jff.2015.05.009

De Champs, C., Maroncle, N., Balestrino, D., Rich, C., & Forestier, C. (2003). Persistence of colonization of intestinal mucosa by a probiotic strain, Lactobacillus casei subsp. rhamnosus Lcr35, after oral consumption. *Journal of Clinical Microbiology*, *41*(3), 1270–1273. https://doi.org/10.1128/JCM.41.3.1270-1273.2003

Defined, C., & Facts, C. (n.d.). Healthier Eating Guidelines for Lowering Cholesterol. Retrieved from http://www.cvtoolbox.com/downloads/diets/high_cholesterol_eating_plan_2010.pdf

Delgado, S., & Rodrı, J. M. (2007). Safety assessment of two probiotic strains , Lactobacillus coryniformis CECT5711 and Lactobacillus gasseri CECT5714, *103*(Conway 1996), 175–184. https://doi.org/10.1111/j.1365-2672.2006.03225.x

Dambekodi, P. C., & Gilliland, S. E. (1998). Incorporation of cholesterol into the cellular membrane of Bifidobacterium longum. Journal of dairy science, 81(7), 1818-1824.

Furrie, E. (2005, November). Probiotics and allergy. In PROCEEDINGS-NUTRITION SOCIETY OF LONDON (Vol. 64, No. 4, p. 465). CABI Publishing; 1999.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., & Gil, A. (2013). Sources, isolation, characterisation and evaluation of probiotics. *Br J Nutr*, *109 Suppl 2*, S35-50. doi:10.1017/S0007114512004011

Formulas, M. (2008). High Fat Diets for Diet-Induced Obesity Models, (October), 2008–2010.

Fuller, R. (2012). *Probiotics: The scientific basis*: Springer Netherlands.

Gatesoupe, F. (1999). The use of probiotics in aquaculture. *Aquaculture*, *180*(1), 147-165.

Gueimonde, M., & Salminen, S. (2006). New methods for selecting and evaluating probiotics. *Digestive and Liver Disease*, *38*, S242-S247. doi:10.1016/s1590-8658(07)60003-6

Reference

Gogineni, V. K. (2013). Probiotics: History and Evolution. *Journal of Ancient Diseases & Preventive Remedies*, *01*(02), 1–7. https://doi.org/10.4172/2329-8731.1000107

Hill, C., & Gahan, C. G. M. (2006). Bile Salt Hydrolase Activity in Probiotics, *72*(3), 1729–1738. https://doi.org/10.1128/AEM.72.3.1729

Hu, X., Wang, T., Li, W., Jin, F., & Wang, L. (2013). Effects of NS lactobacillus strains on lipid metabolism of rats fed a high-cholesterol diet, 1–12.

Han, W., Wang, J., Liu, S., Ge, C., Cao, S., Song, B., … Zhang, X. (2017). Spectral properties of spherical boron nitride prepared using carbon spheres as template. *Ceramics International*, *43*(4), 3569–3575. https://doi.org/10.1016/j.ceramint.2016.11.192

Hu, X., Wang, T., Li, W., Jin, F., & Wang, L. (2013). Effects of NS lactobacillus strains on lipid metabolism of rats fed a high-cholesterol diet, 1–12.

Johansen, K. (1990). Hyperlipidemia in diabetes mellitus: Pathogenesis, diagnosis, and pharmacological therapy. *Annals of Saudi Medicine*, *10*(2), 194–201. https://doi.org/10.5144/0256-4947.1990.194

Journal, W. (2014). In vivo Testing of Functional Properties of Three Selected Probiotic Strains, (December 2005). https://doi.org/10.1007/s11274-005-5741-8

Koslik, H. J., Meskimen, A. H., & Golomb, B. A. (2017). Physicians' Experiences as Patients with Statin Side Effects: A Case Series. *Drug Safety - Case Reports*, *4*(1), 3. https://doi.org/10.1007/s40800-017-0045-0

Ks, S., Krishnappa, S., & Bhardwaj, P. (2013). Safety concerns of Probiotic use : A review, *12*(1), 56–60.

Kumar, M., Nagpal, R., Kumar, R., Hemalatha, R., Verma, V., Kumar, A., … Yadav, H. (2012). Cholesterol-Lowering Probiotics as Potential Biotherapeutics for Metabolic Diseases, *2012*. https://doi.org/10.1155/2012/902917

Kimoto, H.; Ohmomo, S.; Okamoto, T. Cholesterol Removal from Media by Lactococci. *J. Dairy Sci.* **2002**, *85*, 3182-3188.

Reference

Koslik, H. J., Meskimen, A. H., & Golomb, B. A. (2017). Physicians' Experiences as Patients with Statin Side Effects: A Case Series. *Drug Safety - Case Reports*, *4*(1), 3. https://doi.org/10.1007/s40800-017-0045-0

Kumar, M., Nagpal, R., Kumar, R., Hemalatha, R., Verma, V., Kumar, A., … Yadav, H. (2012). Cholesterol-Lowering Probiotics as Potential Biotherapeutics for Metabolic Diseases, *2012*. https://doi.org/10.1155/2012/902917

Lavanya, B., Sowmiya, S., Balaji, S., & Muthuvelan, B. (2011). Screening and characterization of Lactic Acid Bacteria from fermented milk. *British Journal of Dairy Sciences*, *2*(1), 5–10.

Lee, L., Ferguson, H., Bollinger, T., Leighton, F. A., & Meyer, F. (1996). Western College of Veterinary Medicine Health and Disease in Wild Freshwater Fish, *40*(1), 195–211. https://doi.org/10.1016/j.pop.2012.11.003.Hyperlipidemia

Lee, Y. K., & Puong, K. Y. (2002). Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. *Br J Nutr, 88 Suppl 1*, S101-108. doi:10.1079/BJN2002635

Liong, M. T., & Shah, N. P. (2006). Effects of a Lactobacillus casei synbiotic on serum lipoprotein, intestinal microflora, and organic acids in rats. Journal of dairy science, 89(5), 1390-1399.

Liong MT, Shah NP (2005a). Acid and Bile Tolerance and Cholesterol Removal Ability of Lactobacilli Strains. J. Dairy Sci. 88:55-66

Liong, M. T., & Shah, N. P. Ã. (2005). Bile salt deconjugation ability , bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains, *15*, 391–398. https://doi.org/10.1016/j.idairyj.2004.08.007

Lye, H., Rusul, G., & Liong, M. (2010). Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. *Journal of Dairy Science*, *93*(4), 1383–1392. https://doi.org/10.3168/jds.2009-2574

Matos, S. L., De Paula, H., Pedrosa, M. L., Dos Santos, R. C., De Oliveira, E. L., Júnior, D. A. C., & Silva, M. E. (2005). Dietary models for inducing hypercholesterolemia in rats. *Brazilian*

Reference

Archives of Biology and Technology, *48*(2), 203–209. https://doi.org/10.1590/S1516-89132005000200006

Meyer-Ficca, M & Kirkland, J. (2016). Niacin. *Adv Nutr*, *7*(1), 556–558. https://doi.org/10.3945/an.115.011239.TABLE

Modifica-, R. F., & Cyril, W. C. (2008). Practice CMAJ Fact Sheet Fish oil and omega-3 fatty acids, *178*(2), 2008.

Miremadi, F., Ayyash, M., Sherkat, F., & Stojanovska, L. (2014). Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic Lactobacilli and Bifidobacteria. Journal of Functional Foods, 9, 295-305.

Miremadi, F., Ayyash, M., Sherkat, F. and Stojanovska, L. (2014) Cholesterol Reduction Mechanisms and Fatty Acid Composition of Cellular Membranes of Probiotic Lactobacilli and Bifidobacteria. Journal of Functional Foods, 9, 295-305

Nguyen, T. D. T., Kang, J. H., & Lee, M. S. (2007). Characterization of Lactobacillus plantarum PH04 , a potential probiotic bacterium with cholesterol-lowering effects, *113*, 358–361. https://doi.org/10.1016/j.ijfoodmicro.2006.08.015

Nocianitri, K. A., Antara, N. S., Sugitha, I. M., Sukrama, I. D. M., Ramona, Y., & Sujaya, I. N. (2017). The effect of two Lactobacillus rhamnosus strains on the blood lipid profile of rats fed with high fat containing diet. *International Food Research Journal*, *24*(2), 795–802. https://doi.org/10 795-802

Of, R., Death, P., & To, D. U. E. (2018). 29% 8%, 2018.

Ooi, L., & Liong, M. (2010). Cholesterol-Lowering Effects of Probiotics and Prebiotics : A Review of in Vivo and in Vitro Findings, 2499–2522. https://doi.org/10.3390/ijms11062499

Pakes Brogden RN, Heel RC, Speight TM, Avery GS., G. E. (1981). Triazolam: a review of its pharmacological properties and therapeutic efficacy in patients with insomnia. *Drugs*, *22*(2), 81–110. https://doi.org/10.2165/00003495-198122020-00001

Reference

Pereira, D. I. A., & Gibson, G. R. (2002). Cholesterol Assimilation by Lactic Acid Bacteria and Bifidobacteria Isolated from the Human Gut, *68*(9), 4689–4693. https://doi.org/10.1128/AEM.68.9.4689

Plaque, T. Y. (n.d.). Soluble Fibers.

Ramkumar, S., Raghunath, A., & Raghunath, S. (2016). Statin therapy: Review of safety and potential side effects. *Acta Cardiologica Sinica*, *32*(6), 631–639. https://doi.org/10.6515/ACS20160611A

RIVIN, A. U. (1962). Hypercholesterolemia. Use of niacin and niacin combinations in therapy. *California Medicine*, *96*(4), 267–269.

Sedláčková, P., Horáčková, Š., Shi, T., Kosová, M., & Plocková, M. (2015). Two different methods for screening of bile salt hydrolase activity in Lactobacillus strains. *Czech Journal of Food Sciences*, *33*(1), 13–18. https://doi.org/10.17221/299/2014-CJFS

Lye, H.S.; Rusul, G.; Liong, M.T. Mechanisms of Cholesterol Removal by Lactoballi Under

Conditions That Mimic the Human Gastrointestinal Tract. *Int. Dairy J.* **2010**, *20*, 169-175.

Lavanya, B., Sowmiya, S., Balaji, S., & Muthuvelan, B. (2011). Screening and characterization of Lactic Acid Bacteria from fermented milk. *British Journal of Dairy Sciences*, *2*(1), 5–10.

Liong, M. T., & Shah, N. P. Ã. (2005). Bile salt deconjugation ability , bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains, *15*, 391–398. https://doi.org/10.1016/j.idairyj.2004.08.007

Lye, H., Kuan, C., Ewe, J., Fung, W., & Liong, M. (2009). The Improvement of Hypertension by Probiotics : Effects on Cholesterol , Diabetes , Renin , and Phytoestrogens, 3755–3775. https://doi.org/10.3390/ijms10093755

Lye, H., Rusul, G., & Liong, M. (2010). Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. *Journal of Dairy Science*, *93*(4), 1383–1392. https://doi.org/10.3168/jds.2009-2574

Reference

Nguyen, T. D. T., Kang, J. H., & Lee, M. S. (2007). Characterization of Lactobacillus plantarum PH04 , a potential probiotic bacterium with cholesterol-lowering effects, *113*, 358–361. https://doi.org/10.1016/j.ijfoodmicro.2006.08.015

Nocianitri, K. A., Antara, N. S., Sugitha, I. M., Sukrama, I. D. M., Ramona, Y., & Sujaya, I. N. (2017). The effect of two Lactobacillus rhamnosus strains on the blood lipid profile of rats fed with high fat containing diet. *International Food Research Journal*, *24*(2), 795–802. https://doi.org/10 795-802

Ooi, L., & Liong, M. (2010). Cholesterol-Lowering Effects of Probiotics and Prebiotics : A Review of in Vivo and in Vitro Findings, 2499–2522. https://doi.org/10.3390/ijms11062499

Ouwehand, A. C., & Salminen, S. (2003). In vitro adhesion assays for probiotics and their in vivo relevance: a review. *Microbial Ecology in Health and Disease*, *15*(4), 175-184. doi:10.1080/08910600310019886

Parvez, S., Malik, K. A., Ah Kang, S., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of applied microbiology*, *100*(6), 1171-1185.

Patel, A., Prajapati, J. B., & Nair, B. M. (2012). Methods for isolation , characterization and identification of probiotic bacteria to be used in functional foods. *International Journal of Fermented Foods*, *1*(1), 1–13. https://doi.org/10.1017/S0952523800006246

Pereira, D. I. A., & Gibson, G. R. (2002). Cholesterol Assimilation by Lactic Acid Bacteria and Bifidobacteria Isolated from the Human Gut, *68*(9), 4689–4693. https://doi.org/10.1128/AEM.68.9.4689

Polak-Berecka, M., Waśko, A., Skrzypek, H., & Kreft, A. (2013). Production of exopolysaccharides by a probiotic strain of *Lactobacillus rhamnosus* : Biosynthesis and purification methods. *Acta Alimentaria*, *42*(2), 220–228. https://doi.org/10.1556/AAlim.42.2013.2.9

Ramkumar, S., Raghunath, A., & Raghunath, S. (2016). Statin therapy: Review of safety and potential side effects. *Acta Cardiologica Sinica*, *32*(6), 631–639. https://doi.org/10.6515/ACS20160611A

Reference

Rottava, I., Batesini, G., Silva, M. F., Lerin, L., De Oliveira, D., Padilha, F. F., et al. (2009).Xanthan gum production and rheological behavior using different strains ofXanthomonas sp. Carbohydrate Polymers, 77, 65–71.

Saikia, D., Kumar, A., Deka, B., Roy, R., Gupta, K., Namsa, N. D., … Mandal, M. (2017). ScienceDirect Hypocholesterolemic activity of indigenous probiotic isolate Saccharomyces cerevisiae ARDMC1 in a rat model. *Journal of Food and Drug Analysis*, *26*(1), 154–162. https://doi.org/10.1016/j.jfda.2016.12.017

Sedláčková, P., Horáčková, Š., Shi, T., Kosová, M., & Plocková, M. (2015). Two different methods for screening of bile salt hydrolase activity in Lactobacillus strains. *Czech Journal of Food Sciences*, *33*(1), 13–18. https://doi.org/10.17221/299/2014-CJFS

Shokryazdan, P., Jahromi, M. F., & Liang, J. B. (2016). Safety Assessment of Two New Lactobacillus Strains as Probiotic for Human Using a Rat Model, 1–17. https://doi.org/10.1371/journal.pone.0159851

Singh, T. P., Malik, R. K., & Katkamwar, S. G. (2015). Hypocholesterolemic effects of Lactobacillus reuteri LR6 in rats fed on high-cholesterol diet Hypocholesterolemic effects of Lactobacillus reuteri LR6 in rats fed on, *7486*(October 2017). https://doi.org/10.3109/09637486.2014.953450

Soccol, C. R., Porto, L., Vandenberghe, D. S., Spier, M. R., Bianchi, A., Medeiros, P., & Yamaguishi, C. T. (2013). The Potential of Probiotics : A Review, *48*(4), 413–434.

Status, T. S. (1999). Probiotic review. *Food Technology*, *53*(11), 67–77.

Shah, T. Z., Ali, A. B., Jafri, S. A., & Qazi, M. H. (2013). Effect of nicotinic acid (vitamin B3or niacin) on the lipid profile of diabetic and non - Diabetic rats. *Pakistan Journal of Medical Sciences*, *29*(5), 1259–1264.

Singer, P. (2011). [Omega-3 fatty acids as an option in the treatment of NAFLD]. *MMW Fortschritte Der Medizin*, *153 Suppl*(1), 90–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22244020%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/22184802

Song, D., Hayek, S., & Ibrahim, S. (2012). Recent application of probiotics in food and agricultural science. INTECH Open Access Publisher.

Sanders, M. E. (2003). Probiotics: considerations for human health. *Nutrition reviews, 61*(3), 91-99.

Singh, T. P., Malik, R. K., & Katkamwar, S. G. (2015). Hypocholesterolemic effects of Lactobacillus reuteri LR6 in rats fed on high-cholesterol diet Hypocholesterolemic effects of Lactobacillus reuteri LR6 in rats fed on, *7486*(October 2017). https://doi.org/10.3109/09637486.2014.953450

Song, D., Ibrahim, S., & Hayek, S. (2012). Recent Application of Probiotics in Food and Agricultural Science, 3–36.

Song, W.-L., & FitzGerald, G. A. (2013). Niacin, an old drug with a new twist. *Journal of Lipid Research*, *54*(10), 2586–2594. https://doi.org/10.1194/jlr.R040592

Silva, M. L. C., Martinez, P. F., Izeli, N. L., Silva, I. R., Vasconcelos, A. F. D., Cardoso, M.S., et al. (2006). Caracterizac¸ ão Química de Glucanas Fúngicas e Suas Aplicac¸ õesBiotecnológicas. Quimica Nova, 29(1), 85–92

Tanaka, H., Doesburg, K., Iwasaki, T., & Mierau, I. (1999). Screening of Lactic Acid Bacteria for Bile Salt Hydrolase Activity. *Journal of Dairy Science*, *82*(12), 2530–2535. https://doi.org/10.3168/jds.S0022-0302(99)75506-2

Tahri, K., Crociani, J., Ballongue, J., & Schneider, F. (1995). Effects of three strains of bifidobacteria on cholesterol. Letters in applied microbiology,21(3), 149-151.

Tomaro-duchesneau, C., Jones, M. L., Shah, D., Jain, P., Saha, S., & Prakash, S. (2014). Cholesterol Assimilation by Lactobacillus Probiotic Bacteria : An In Vitro Investigation, *2014*.

Tuomola, E., Crittenden, R., Playne, M., Isolauri, E., & Salminen, S. (2001). Quality assurance criteria for probiotic bacteria. *The American journal of clinical nutrition*, *73*(2), 393s-398s.

Tomaro-duchesneau, C., Jones, M. L., Shah, D., Jain, P., Saha, S., & Prakash, S. (2014). Cholesterol Assimilation by Lactobacillus Probiotic Bacteria : An In Vitro Investigation, *2014*.

Usman, H.A. Bile Tolerance, Taurocholate Deconjugation, and Binding of Cholesterol by

Reference

Lactobacillus gasseri Strains. *J. Dairy Sci*. **1999**, *82*, 243-248.

UK Factsheet. (2018). *British Heart Foundation*, (August). https://doi.org/10.1016/j.partic.2008.03.004

Weitz, D., Weintraub, H., Fisher, E., & Schwartzbard, A. (2011). NIH Public Access, *18*(5), 258–263. https://doi.org/10.1097/CRD.0b013e3181ea0de0.Fish

World Health Oganization. 2014 .Cardiovascular Diseases, Fact sheet N°317, in the Global status report on noncommunicable diseases 2014, Geneva, Switzerland, September, Downloaded from *http:// www.who.int/mediacentre/ factsheets/fs317/en/* on 27/4/2015

Yusuf, S., Hawken, S., Ôunpuu, S., Dans, T., Avezum, A., Lanas, F., … Pais, P. (2004). Yusuf et al 2004-Interheart, 937–952. https://doi.org/10.1016/S0140-6736(04)17018-9

Ziemer, C. J., & Gibson, G. R. (1998). An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. International Dairy Journal, 8(5), 473-479

Zubair, F., Nawaz, S. K., Nawaz, A., Nangyal, H., Amjad, N., & Khan, M. S. (2018). Prevalence of cardiovascular diseases in Punjab , Pakistan : a cross-sectional study.

Muneera A, (2017) The assessment of probiotic properties of indigenous *Lactobacillus* strains with GABA producing potential(unpublished) NUST, Pakistan, Islamabad.