Isolation and characterization of *Lactobacillus* strains and evaluation of their antibacterial and cytotoxic activities

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By

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MS THESIS WORK

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DECLERATION

I, **ARSLAN AMER**, declare that all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work herein was carried out while I was postgraduate student at Atta-ur-Rahman school of Applied Biosciences-NUST under the supervision of Dr. Rumeza Hanif.

I would like to dedicate

this thesis to

DAENARYS STORMBORN

of House Targaryen of Blood of Old Valyria

Queen of the Andals, Rhoynar and the First Men

Queen of Mereen, Astapor and Yunkai

The Khaleesi of Great Grass Sea

Queen of the seven kingdoms

And protector of the realm

The Unburnt, Breaker of Chains

and Mother of Dragons

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The aforesaid project has been reviewed by Institutional Review Board (IRB) Committee, ASAB, keeping in view the following selection criteria:

- Qualification, Expertise and Scientific Caliber of the Principal Investigators
- Proposed Goals of the Study
- Subject Selection
- Selection Criteria of Subjects
- Informed Consent Process
- Potential Problems
- Research Design and Methods
- Potential Benefits of the Study
- Risks of the Study
- Management of Risks
- Assessment of Risk
- Confidentiality
- Conflict of Interest

The committee thus **APPROVES** the project on "**Safety Assessment of Probiotics on Mice models**" on the scales and criterion set by IRB.

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LIST OF ABBREVIATIONS

AAD	Antibiotic Associated Diarrhea
ACF	Aberrant Crypt Foci
ALT	Alanine Transaminase
AMPs	Antimicrobial Peptides
CTX-30	Cefotaxime-30 µg.
CXCL	C-X-C motif ligand
EFGR	Epidermal growth factor receptor
F6PPK	Fructose6 Phosphate Phosphoketolase
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GIT	Gastrointestinal Tract
GRAS	Generally Recognized as Safe.
GSH	Glutathione
GST	Gluthathione S-Transferase
HCC	Hepatocellular carcinoma
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IECs	Intestinal Epithelial Cells
IL-6	Interleukin 6
LAB	Lactic Acid Bacteria
LMW	Low Molecular Weight
LPS	Lipopolysaccahride
MRS Agar	de Man Rogosa Sharpe Agar
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic Steatohepatitis
NEC	Necrotizing Enterocolitis

NFkB	Nuclear Factor Kappa Beta
NLR	Nod like Receptors
NO	Nitrogen oxide
PLB	Probiotic Lactic Acid Bacteria
PBS	Phosphate Buffer Saline
SS Agar	Salmonella Shigella Agar
STEC	Shiga Toxin *Escherichia coli*
TGF	Transforming Growth factor
TLR	Toll like Receptors
TNF	Tumor Necrosis Factor
TOS	Transgalctosylated Oligiosaccharide
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infection
WHO	World Health Organization

ABSTARCT

Probiotics are considered to be living organisms. Which administer some kind of a health effect on their host, when present in a significant amount. Many different genera of bacteria have contributed to probiotics, but Lactic Acid Bacteria (LAB) are of prime importance. studies have shown that probiotic bacteria can restore the gut flora and inhibit pathogens. Currently probiotic role in cancer treatment is also being considered. The aim of the present study was to isolate and characterize LAB strains from local indigenous sources and to evaluate them as potential probiotics. Duck intestines were used as sources for isolation of these bacteria. Identification of bacterial strains was performed by biochemical testing and Ribotyping. Survival, adhesion and colonization were examined in gastrointestinal tract of mice. Antimicrobial activity of isolates against *STEC* and *Shigella* was evaluated by well diffusion assay. Cytotoxic activities against cancerous cell lines were evaluated using MTT colometric assay. Results of biochemical testing and riboprinting revealed that isolates belong to *Lactobacillus Plantarum*. Rifampicin-resistant colonies of these strains were able to be re-isolated from the faeces of mice which were fed these probiotics ensuring their survival. The similar results of colonization were obtained in homogenates of small intestine; large intestine and caecum of mice on the twenty-eight day after feeding with the selected Strains. Results of antibacterial testing showed that each strain of the probiotic bacteria had varying activity against the pathogens under similar conditions. In addition, our findings showed a significant cytotoxic activity against HeLa cell lines. The positive results of the study suggest that these *L.Plantarum* strains may be useful as potential probiotic candidates.

1 INTRODUCTION

The intestinal microbiota is composed of several bacterial species and a variety of strains that help utilize nutrients present in food and play a significant role in improving development and performance of immune system (Sanders, 1999). These beneficial microbes are called probiotics.

Fuller defined probiotics as: 'A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 2001). FAO/WHO in 2001 gave the most generalized explanation of probiotics, as the viable cells which when given in appropriate amounts have beneficial health effects (FAO/WHO, 2001) The word probiotics has been first used in 1974 but history of probiotics in domestic uses dates back to thousands of years.

Throughout history probiotics have been used by mankind in the form of functional foods. The Old Testament reported that fermented milk was the first food that contained probiotics (Reviewed in Fuller, 1989). In fermented foods such as yogurt and other dairy products probiotics are present as live cultures and offer a rich source of probiotic bacteria (Salminen *et al.,* 1998). Majorly sources of probiotics are considered to be milk and milk based products but they also have been isolated from gut of various animals including humans. Breast milk, meat and fruits (Loing, 2001; Solis *et al.,* 2010).

For a strain to be classified as probiotic, it should be safe, stable, non-pathogenic and capable of exerting beneficial effects on humans. It is also perceived to exhibit a certain health benefit to host. The probiotics are known to be helpful against certain common health problems such as Irritable Bowel Syndrome (IBS), Inflammatory Bowel Disease (IBD), diarrhea of different etiologies and lactose intolerance. Probiotics also have antibacterial properties against food-borne pathogens

including *Campylobacter spp., Clostridium botulinum, Listeria monocytogenes, Escherichia coli* and *Shigella* (Delley *et al.,* 2015; Srividya *et al.,* 2015).

There are several mechanisms of action proposed for antibacterial activities of probiotics. Probiotics may function by enhancing the epithelial barrier through upregulating the genes involved in tight junction signaling (Anderson *et al.,* 2010), by preventing the cytokine induced epithelial damage (Yan *et al.,* 2002) (Yan *et al.,* 2007) and/or by increasing mucus secretion (Kim *et al.,* 2008). Some probiotic bacteria inhibit the pathogens by competing for nutrients and receptor sites (Fujiwara *et al.,* 2001). Probiotics also release antimicrobial substances such as organic acids and bacteriocins to kill the pathogenic organisms. Probiotics are also known to have immuno-modulatory properties, they interact with Toll Like Receptors (TLRs) and Nod Like Receptors (NLRs) to regulate cellular signaling pathways resulting in the inhibition of harmful organisms (Castillo *et al.,* 2011).

Recently, significance of probiotics in cancer has become the burning area of research. Many epidemiological evidences have surfaced that support the hypothesis that probiotics play a protective role against cancer (Goldin, 1996; Kampman, 1994; Hirayama, 2000). A number of Lactic Acid Bacteria (LAB) have found to be involved in prevention of cancer, such as *B. longum, L. casei, L. acidophilus, B. lactis,*and *B. Infantis* (Haskard *et al*., 2000; Matsumoto *et al*., 2009; Vanderhoof, 2001; Biffi *et al*., 1997; Femia *et al*.,2002). Different strains of health promoting bacteria and fungi exhibit effects that help in the inhibition of mutagenic activity and reduce the concentration of enzymes that generate carcinogens and tumour promoting agents (Fernandes and Shahani, 1990; Marteau *et al*., 1990). Regulation of tumour necrosis factor, augmentation of cytokines and interleukins, and alteration in immune response are induced by some specific components of LAB (Erickson and Hubbard, 2000; Matsuzaki *et al*. 2007; Takagi *et al*. 2008).

Few recent *in-vivo* and *in-vitro* studies have shown that probiotics can prove to be helpful in preventing several cancers, including liver, colon and breast (Haghshenas *et al*., 2015; Kahouli *et al*., 2015). Several mechanisms have proposed probiotics as anticarcinogen agents Many of the underlying mechanisms are likely to be multifactorial, but are yet to be studied. Several chronic diseases, including cancer, have known to be associated with oxidative stress. A study conducted by Ahotupa *et al*., in 1996 showed that some strains of probiotics are scavengers of superoxide anion, thus prevent cancer by reducing the oxidative stress (Ahotupa*et al*. 1996). Moreover, recent studies show that LAB and other intestinal bacteria absorb or bind to several carcinogens, decreasing their mutagenicity (Caldini *et al*. 2005; Chalova *et al*. 2008).

What makes research in probiotics interesting, is its strain specific characteristics. It has been widely reported that characteristics necessary for an isolate to be considered a potential probiotic are strain specific and vary greatly between species. This has led to speculation that there may be many unknown strains present in nature with undiscovered potential.

In this context, the present study intends to

- Isolate and characterize indigenous Lactic Acid Bacterial strains.
- Evaluate the functionality of strains by monitoring their survival and colonization in mice gastrointestinal tract
- Explore the anti-microbial properties of probiotic cell free supernatant against food-borne bacteria such as *STEC* and *Shigella*.
- Evaluate the anti-carcinogenic activity of different strains of *isolated strains* against cancerous cell lines.

2 LITERATURE REVIEW

The term "probiotics" was first introduced by Lilley and Stillwell in 1965 as an opposite of the word "Antibiotic". They defined "probiotics" as substances which favor the growth of microorganisms (Saavedra & Jose, 1965). The definition was revised over the period of time as new researches emerged out and suggested new characteristics, criteria for and application of probiotics. The latest definition of probiotics by WHO also emphasizes on the quantity of intake of probiotics; it states, "probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host." (FAO/WHO, 2012)

2.1 History of Probiotics:

Although the word "probiotics" appeared in the 20th century, the history of live microbial feed supplements (including probiotics) dates back to thousands of years. It is assumed that the first foods that contain probiotics were fermented milk (Fuller & Roy., 1992). Fermented milk has been consumed in different forms to provide health benefit. Metchnikoff, on the basis of his studies, advocated the use of milk fermented with a particular Lactobacillus strain for longevity (Rettger., 1917). The interest in probiotic studies saw periods of decline and rise in the subsequent years. Rettger and his colleagues, in 1921, used intestinal isolates of *Bulgarican bacillus* in their studies (reference). In 1935, Rettger and colleagues again used the probiotic strain, L. acidophilus, in their experiments for therapeutic purposes (Rettger et al., 1935). However, at this stage the studies conducted were quite preliminary. The general belief about bacteria being harmful to the body existed widely. Significant contribution for establishing the beneficial properties of probiotics was done by Bohnhoff (Bohnhoff et al., 1954) and Freter (Freter & Rolf, 1955), their studies demonstrated that the administration of antibiotics in mice can make them more susceptible to

infections by certain bacteria (such as Salmonella, Shigella, Vibrio). The restoration of the gut microflora by administration of fecal suspensions was found successful to treat gut infections.

Today it is a well-known fact that animals have certain bacteria in their gut which naturally function to protect them against diseases. Yet, this population of naturally occurring flora is affected by modern methods of perinatal care, diet, stresses and an intake of antibiotic drugs in humans. The purpose of taking probiotics in foods and as supplements is to restore the otherwise natural microbial balance (Fuller, 2012). Probiotics also present themselves as a candidate alternative for antibiotics whose side effects are unwanted and whose efficacy is decreasing due to drug resistance (Reid & Gregor, 2006).

2.2 Sources of Probiotic Bacteria

Traditionally milk and dairy products are considered the best sources of probiotic bacteria. There is a long history of spontaneous milk fermentation in Africa and Mongolia which has been used for obtaining LAB and other microorganisms for centuries (Yu *et al.,* 2011).

148 LAB strains were isolated from Kurut, which is a traditional fermented yalk milk from China, among those most dominant isolated strains were L. *delbrueckii subsp*, *bulgaricus* and *Streptococcus thermophilus* (Sun *et al.,* 2010). Probiotic yeast and lactobacilli strains were isolated from kefir grains, masai milk and koumiss these strains were shown to modulate immune response (Lopitz *et al.,* 2006; Patrignani *et al.,* 2006).

Traditional fermented milk was studied for its probiotic composition that indicated that most of the organisms present in fermented milk belonged to lactobacillus genus (Vizoso *et al.,* 2006). Cheese is an important source for delivery of probiotics to the human gut. Italian, Argentinean and Bulgarian cheeses are rich sources of *L. planturum* strain (Zago *et al.,* 2011). Breast milk is also a

rich source of various LAB strains, these strains found in the breast milk were observed in the faeces of breast fed infant (Martin *et al.,* 2003). Breast fed infants have low risk of developing allergies and intestinal diseases than that of formula fed infants thus proving a better intestinal microbiota in breast fed infants (Solis *et al.,* 2010). Predominant bacteria in the breast milk of humans are *Staphylococci*, *Streptococci*, *Micrococci*, *Lactobacilli*, *Enterococci*, *Lactococci* and *Bifidobacteria* (Reviewed in Luis *et al.,* 2013) and consumption of breast milk causes predominance of *Bifidobacterium* and *Lactobacilli* in infants' intestine. Infectious mastitis can be caused during lactation which can be effectively treated by *Lactobacilli* isolated from breast milk (Jime *et al.,* 2013) and these strains are able to enhance both innate and acquired immune responses.

Latest Research has focused on the gut of animal species including rats and poultry as a source of probiotics (Petrof , 2009). Probiotics have been isolated from the intestine of marine and fresh water fish (Reviewed in Luis *et al.,* 2013). Some probiotic strains have been isolated from meat and fruits similar to that of intestinal bacteria (Haller *et al.,* 2001). Isolation of probiotic strains from brine and pickled juices have also been reported (Abriouel *et al.,* 2011).

2.3 Characteristics of Probiotic Bacteria:

In order for bacteria to be efficacious as a probiotic, it must harbor certain characteristics. The probiotic bacteria are expected to survive in the intestinal ecosystem, it should have the necessary adaptations to reach the intestines through the highly acidic environment gastrointestinal tract (GIT) It is also expected to colonize and thrive in the intestines and exert a beneficial effect on the host who harbors it.

2.3.1 Acid and Bile Tolerance

Probiotic organisms are administered orally, which means they have to pass through the stomach albeit transiently. Many yoghurt producing bacteria like L. delbrueckii subsp. bulgaricus and S. thermophilus often do not survive the harsh stomach pH, which can drop to even 1.5 in fasting individuals. Similarly, the bacterium must also be tolerant of bile salts present in the intestines.

Clark *et al.* (1993) and Lankaputhra and Shah (1995) showed that not all the strains of lactobacillus and bifidobacteria are tolerant of acidic and bile environments. They have showed that tolerance of low pH and bile salt is strain dependent. And even different strains of the same specie behave differently under these conditions.

2.3.2 Adhesion and colonization

Adhesion is the most significant interaction between the host and probiotic strain and it is also important for colonization to the intestinal mucosa (Schiffrin *et al.,* 1997). It is important for immunomodulation (Perdigon *et al.,* 2003) and competitive exclusion of pathogenic organisms (Hirano *et al.,* 2003). This property is one of the standards for probiotic strains selection (Crociani *et al.,* 1995). LAB attach with the intestinal epithelial cells with the help of surface determinants. Mucin is secreted by intestinal epithelial cells which is a complex glycoprotein that prevents adhesion of pathogenic bacteria (Collado *et al.,* 2005; González *et al.,* 2012). Mucus gel also contains lipids, free proteins, immunoglobulins and salts (Neutra *et al.,* 1987). The relationship between surface proteins of probiotics and competitive exclusion of pathogens from the mucus can be explained by the interaction of these surface proteins with mucin (Van *et al.,* 2011). Most popular bacterial adhesion protein is mucus binding protein (MUB) synthesized by *lactobacillus reuteri* (Buck *et al.,* 2005)

Surface proteins are found to interact with human plasmogen or enterocytes in *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp Lactis, respectively. These proteins facilitate colonization of gut of human by aiding epithelial contact and through degradation of extracellular matrix of cells (Sánchez *et al.,* 2010) for example *L. reuteri* and *L. fermentum* attachment to mucus is reported to be facilitated by Map A (mucous adhesion-promoting protein) (Ouwehand *et al.,* 2002).

Collado *et al.* showed that acid resistant strains have greater stability to adhere to intestinal mucus. Probiotics strains can cause qualitative changes in mucin that inhibits pathogen binding (Collado *et al.,* 2006). Defensins are released from probiotic strains that have activity against microorganisms including bacteria, fungi and viruses. They also help to improve gut barrier function (Furrei *et al.,* 2005). As a reaction to pathogenic attack, host produces several antimicrobial proteins such as defensins, cathelicidins, C-type lectins and ribonucleases that provides first line chemical defence (Ganz *et al.,* 2003; Gallo *et al.,* 2012). Adhesion of LAB involves passive, electrostatic, hydrophobic and steric forces, lipoteichoic acids and external accessory structures coated by lectins. Molecules involved in adhesion of pathogenic microbes have been widely characterized however, studies characterizing the factors that mediate lactobacillus adhesion are still limited (Miriam *et al.,* 2012).

2.3.3 Safety

In order for a microbe to be deemed as a probiotic, its safety to the consumer should be tested. If capable of causing pathogenicity, the bacteria cannot be considered as a probiotic.

For assessment of safety of probiotics, following factors needs to be considered.

(1) Determining isolation history and taxonomy of microbe,

(2) manufacturing and storage controls,

(3) strain level assessment of infectivity and toxicity,

(4) considering the vulnerability (for new born and seriously ill patients) and physiological status of consuming population.

If a strain successfully pass though all these factors it can be regarded as safe but the broad assumption can still not be made (Luis *et al.,* 2013).

2.3.4 Beneficial Effects

From the second half of the definition of probiotics as described by WHO in 2002 a probiotic should "confer some health benefit". These beneficial effects can range from better nutrient absorption to ward of different diseases and health conditions.

2.3.4.1 Antibiotic Associated Diarrhea (AAD)

Diarrhea that occurs shortly after the intake of an antibiotic is called Antibiotic Associated Diarrhea. (AAD) has a higher prevalence in adults as compared to children (Muldoon et al., 2014). AAD results in prolonged hospital stays and increased costs. Antibiotics e.g. Aminopenicillins, Cephalosporins, and Clindamycin lower the concentration of anaerobic microorganisms in the intestine. This results in decreased carbohydrate metabolism leading to osmotic diarrhea and overgrowth of enteric pathogens (Muldoon et al., 2014).

Studies conducted by Wenus *et al.,* (2008) and Hickson *et al.,* (2007) showed that there is a lower incidence of AAD in the patients receiving probiotics. Similar studies were conducted by Kotowska *et al.,* (2005) and Gao *et al.,* (2010) and showed a positive role of probiotics in improving AAD. Probiotic strains that have been studied for AAD *are Lactobacillus acidophilus, L. bulgaricus, Lactobacillus casei, L. rhamnosus, B. bifidum, B. longum, S. thermophilus, S. boulardii and Clostridium butyicum* (Muldoon et al., 2014).

McFarland administered probiotics to study the effect on AAD in both children and adults (McFarland, 2006). 44% of the trials showed efficacy of probiotics in adults whereas 67% of trials showed beneficial effects in children. In another trial (Hickson *et al*., 2007) yoghurt drink product Actimel® containing L. casei DN-114 001, *Streptococcus thermophilus* and L. bulgaricus was used. A significant reduction in both the incidence of AAD and C. difficle Associated Diarrhea (CDAD) was observed. The data for the role of probiotics for treating AAD in children is also promising especially for Lactobacillus GG and S. boulardii but not quite conclusive (Muldoon *et al*., 2014).

2.3.4.2 Lactose Intolerance:

Lactose Intolerance is characterized by indigestion of milk and milk products resulting in diarrhea, abdominal cramps and flatulence. According to research, it has been shown that if milk is replaced by fermented dairy products such as cheese and yogurt, the symptoms of lactose intolerance can be relieved. The bacterial enzyme called B-glactosidase improves the digestibility of lactose in lactose-intolerant individuals. Yet, the activity of this enzyme among different probiotic strains varies significantly. The probiotic bacteria *L .acidophilus* is reported to alleviate the symptoms of lactose intolerance (Kim *et al.,* 1983)

2.3.4.3 Irritable Bowel Syndrome (IBS)

Irritable Bowel Syndrome is a chronic commonly occurring disease of the GIT. The use of drugs against IBS is not found to be very effective. Probiotics are a popular alternative used against IBS (Hoveyda *et al.,* 2009).

IBS is recognized by bloating, abdominal pain and bowel dysfunction. The people affected by this disorder were reported to have lower quality of life scores. Probiotics when given in the form of food items such as yogurt and other dairy products can be much effective than conventional drugs for treating IBS (Hoveyda *et al.,* 2009). Simren *et al.,* (2006) found that gut bacteria play a role in

the control of gut function. People with IBS have a different combination of gut microflora as compared to healthy people (Stojanović *et al.,* 2011). The changes in gut microflora by the administration of probiotics produce healthy effects in patients with IBS (Simren *et al.,* 2006; Moayyedi *et al.,* 2010). A combination of probiotic bacteria taken on a regular basis also improves the quality of life in IBS patients (Zúñiga *et al.,* 2014).

2.3.4.4 Inflammatory Bowel Disease (IBD):

Inflammatory Bowel Disease is a chronic inflammation of all or some part of the digestive tract. IBD primarily consists of two distinct immune mediated gastrointestinal disorders, Ulcerative Colitis (UC) and Crohn's Disease (CD) (Veerappan *et al.,* 2012). The use of conventional steroids, thiopurines and anti-tumor necrosis factor antibodies to treat IBD is associated with numerous side effects.

Researchers have been trying to propose new therapies for IBD that could possibly restore the balance of commensal bacteria. Probiotics have been explored as an alternative therapy for IBD (Veerappan *et al.,* 2012). Probiotics were found to be effective against the remission of pouchitis (inflammatory condition in the pouch reservoir created in the management of patients with UC). Probiotics are relatively safe to use for IBD with minimal side effects or contraindications.Another study by Whelan *et al.,* (2013) concluded that probiotics through the promotion of mucosal immunoregulation and through their impact on the host gastrointestinal microflora can be effective to treat IBD patients. Probiotics have a number of benefits in pouchitis and UC (Whelan *et al.,* 2013). However, studies demonstrating the role of specific probiotic strains to treat IBD still need to be performed (Whelan *et al.,* 2013).

2.4 Antibacterial Activity

2.4.1 Campylobacter spp

Campylobacter is known as the causative agent of bacterial food borne diarrheal disease. *C. jejuni* and *C.coli* are the most prominent species. *C. coli* is found in pork meat and causes infections in humans whereas *C. jejuni* is found in raw milk, raw meat/poultry/shellfish and untreated water. Bratz *et al.,* (2015) evaluated the inhibitory activity of probiotics against *Campylobacter spp*. Cell free culture supernatants of the probiotic bacterium, *Lactobacillus* showed inhibitory activity against one (of the three tested) Campylobacter strains. The antimicrobial effect was produced due to the production of organic acids that lowered the pH. The antimicrobial effect of the probiotics was gone when the pH was neutralized (Bratz *et al.,* 2015).

2.4.2 Clostridium botulinum

C. botulinum produces a toxic component called botulinum which causes muscle paralysis (botulism). *C. botulinum* can be found in home-canned and prepared foods, vacuum packed and tightly wrapped foods as well as meat products/seafood and herbal cooking oils (FDA, 2014). Rodgers, Svetlana *et al.,* (2008) added *Lactococcus lactis* ssp. *lactis* to certain sous-vide food products and found that the populations of *Clostridium botulinum* were reduced. The study concluded that these probiotic bacteria can be used in the food industry for food preservation or as a functional ingredient (Rodgers & Svetlana, 2008)

2.4.3 Listeria monocytogenes

Listeria is a pathogenic bacterium that can slowly grow at low temperatures. It is present in refrigerated, ready-to-eat foods (meat/poultry/seafood, dairy unpasteurized milk and milk products). Infections by *Listeria* can lead to miscarriages in pregnant women, diarrhea, meningitis, muscle aches, nausea, fever and fatigue (FDA, 2014). Bendali *et al.,* (2014) isolated probiotic bacteria *Lactobacillus paracasei* subsp. *paracasei* from newborn faeces and checked its effect on

Listeria monocytogenes. It was observed that *Lactobacillus* had an anti-adherence effect on *Listeria.* Moreover, the cell free supernatant of *Lactobacillus* was also checked for its anti-listerial activity. *Lactobacillus* produces bacteriocin-like substances and displaces the pathogen from different surfaces (the surface of Caco-2 cell line and the surfaces of stainless steel and teflon which are used in the food industry) (Bendali *et al.,* 2014).

2.4.4 Escherichia coli

E. coli is a pathogenic group of bacteria which can produce a variety of deadly toxins (FDA, 2014). Infection with *E. coli* leads to Urinary Tract Infection (UTI) and diarrhea (Sharma *et al.,* 2014). The strain of *E. coli,* 0157:H7 causes permanent kidney damage in children which can be fatal (FDA, 2014). Probiotics such as *Lactobacillus rhamnosus, Saccharomyces boulardii, Streptococcus faecalis* and *Lactobacillus acidophilus* are found to have inhibitory effect on *E. coli.* Sharma *et al.,* (2014) showed that probiotics can be used to increase the activity of antibiotics against *E. coli* e.g. *Streptococcus faecalis* given in combination with the antibiotic *Ceftazidime* enhanced the zone of inhibition from 18mm to 24mm against MTCC what is this?? and clinical isolates of *E. coli.* More than 80% of the cases of UTI are caused by uropathogenic strain of *E. coli (E. coli* UPEC). Delley *et al.,* (2015) demonstrated that *Lactobacilli* prevent UTI recurrence by restoring the microbial balance of the urogenital tract. *Lactobacilli* bacteria inhibit UPEC by acidifying the environment of the urogenital tract (Delley *et al.,* 2015).

2.4.5 Shigella

Shigella is transmitted from one person to another through food due to poor hygiene and bad handwashing practices. It is commonly found in salads, milk and dairy products, ground beef, poultry and unclean water (FDA, 2014). Srividya *et al.,* (2015) studied the effect of the probiotic LAB, *Pediococcus pentosaceus,* on *Shigella dysentriae* and observed that probiotic lysate showed

better inhibition of *S. dysentriae* as compared to the antibiotic Ampicillin. Probiotic lysate also showed protection against cellular damage caused by *S. dysentriae.*

3 MATERIALS AND METHODS

The present research was conducted in Atta-ur-Rahman School of Biosciences (ASAB) at National University of sciences and Technology (NUST) to isolate and characterize probiotic strains and also to evaluate their antibacterial and cytotoxic effects. This investigation was arried out by using Lactobacillus strains. All the materials used and analytical methods applied in this research are mentioned below.

3.1 Chemicals

3.1.1 Man, Rogosa and Sharpe Media (MRS)

Man, Rogosa and Sharpe media (MRS) was used for the growth of Probiotic strains (De Man et al., 1960). The media was prepared by adding 1% (w/v) beef extract (Merck, Germany), 0.4% (w/v) Yeast Extract (Merck, Germany), 1% (w/v) Peptone (Merck, Germany), 2.0% (w/v) glucose (Merck, Germany), 0.5% (w/v) Sodium Acetate trihydrate (Sigma Aldrich, Germany), 0.2% (w/v) Triammonium citrate (Sigma Aldrich, Germany), 0.2% (w/v) Di potassium hydrogen phosphate (Merck, Germany), 0.02%(w/v) Magnesium sulphate (Merck, Germany), 0.005% (w/v) Maganese Sulphate tetrahydrate (Merck, Germany) and 0.1 % (v/v) Tween 80. The media was enriched with 0.05% L-cysteine (Sigma Aldrich, Germany). The media was prepared with distilled water and autoclaved.

3.1.2 Phosphate Buffer Saline (PBS)

Phosphate Buffer Saline was made through standard protocol i.e 1.4 mM Potasium phosphate(Merck, Germany), 2.7mM Pottasium Chloride(Merck, Germany), 4.3 mM Sodium Phosphate(Merck, Germany), and 137mM Sodium Chloride(Merck, Germany). The pH was adjusted to 7.2 and sterilized by autoclave. PBS was stored at Room Temperature.

3.1.3 Luria-Bertani Media (LB)

Luria bertani Media was used for the growth of pathogen strains and for antibacterial well diffusion assay. The media was prepared by adding 1%(w/v) Tryptone(Merck, Germany), 1% Sodium Chloride(Merck, Germany), and 0.5% Yeat Extract (Merck, Germany). The media was prepared with distilled water and was autoclaved at 120c for sterilization.

3.1.4 Tris-EDTA buffer Solution

TE buffer was prepared by mixing 10 mM Tris HCl and 1 mM EDTA (pH 8.0). It was then diluted by adding 10 ml buffer (100X) in 990 ml distilled water. Final pH of buffer was to be approximately 8.0 and was stored at room temperature.

3.1.5 1X Tris Acetate Ethylenediaminetetraacetic (TAE)

Tris Acetate EDTA (TAE) was initially prepared as a stock solution of 50X concentration. 1X TAE was used for stabilization and maintaining the pH during agarose gel electrophoresis. For the preparation of 50X TAE, 890 mM of tris base, 890 mM of Acetic acid and 20 mM of EDTA (pH 8.0) was dissolved in distilled water. To achieve the desired volume, more of the distilled water was added. 1X TAE could then be prepared from the stock solution. 1X TAE had 89 mM Acetic acid, 89 mM tris base and 2 mM of EDTA as final concentration.

3.1.6 Rifampicin Solution

Rifampicin stock solution was prepared by dissolving 450 mg of rifampicin (Sigma Aldrich, Germany) to 9ml of Dimethyl sulphoxide (DMSO) (CARL Roth, Karlsruhe , Germany). to achieve the final concentration of 50mg/ml. the solution was syringe filtered and stored at 4 c.

3.2 Sample collection

A month old duckling was first anesthetized with chloroform (AnalaR, England). Then it was dissected by DVM Dr. Alam at animal house facility, NUST). A piece of small intestine was separated by pieces of sterile strings. And then they were cut so that the two mouths were closed shut to prevent oxygen entry. This piece was then washed alternately by distilled water and ethanol and was placed in a sterile container containing PBS (enriched with L-cysteine). The sample was further processed in laminar flow hood. Where it was cut and contents inside were scratched by a sterile swab. These swabs were later streaked on MRS medium enriched with L-Cysteine. And were incubated Anaerobically for 48 hours. Individual colonies were picked and steaked further and glycerol stocks were maintained at -20ºC

3.3 General Characterization and biochemical testing of Probiotics:

Initial characterization was done to ensure lactobacillus presence:

3.3.1 **Gram staining:**

The bacterial strains were streaked on MRS Agar and incubated overnight. A single, pure colony was picked from the streaked plates and spread slightly on a water droplet present on a glass slide (Globe Scientific Inc. USA). The cells were stained with crystal violet dye (Merck Pvt Ltd. Pakistan). Next, Gram's iodine solution (Merck Pvt Ltd. Pakistan) was added followed by the addition of ethanol and Safranin (Merck Pvt Ltd. Pakistan). The slides were observed under a light microscope (Optika Italy) with oil immersion.

3.3.2 pH Survival Assay

Survival of the selected bacterial strains was checked in varying acidic pH range. The isolated colony was inoculated in MRS ranging from pH 5 to 2.5, The pH was adjusted with 6N HCL.

Samples were plated every 30 min and were spread plated to determine Live Bacterial cells in the Broth.

3.4 *In vivo* Probiotic Potential testing

In order for an organism to be classified as a potential probiotic, it is supposed to have certain characteristics like GIT survival, adhesion and colonization of intestinal mucosa. For our experiment we have chosen the in vivo model to determine these characteristics.

3.4.1 Rifampicin Tagging

In order to distinguish the probiotic strains from the microflora of the test animals, selected strains were tagged by inducing Rifampicin resistance. Rifampicin resistant strains were generated by culturing probiotic cultures to MRS agar containing increasing concentration of rifampicin (25, 50, 100 ug/ml). Rifampicin resistant strains were selected and checked for stability by repeat culturing on MRS media supplemented with 200ug/ml of rifampicin for at least 20 generations.

3.4.2 Animal Model

Approval for the experiments concerning animal model was taken from the internal board review (IRB form is attached). A total of 40 Balb/C mice were taken between the ages of 6-8 weeks. The mice were divided into four groups of 5 mice each(two males and three females). The mice were kept in different cages under standard conditions, i.e; 12h light/12h dark cycle, 18-23 c, water accessibility. The mice were fed non-sterile diet (Standard animal feed). 3 were taken as test groups while one group was used as control. Fecal samples of all the animals used in the experiments were checked for Rifampicin resistant strains by incubation of fecal matter on MRS containing 200ug/ml Rifampicin. No bacterial colonies were observed till 48 hours of incubation.

3.4.3 Probiotic Dosage

The probiotic dose was administered to the animals through drinking water. A total dose of 2 billion CFU/mouse/day was given continuously for 24 days, the control was given PBS mixed with water in similar concentration.

3.4.4 Gastrointestinal Transit

To evaluate the survival of bacterial strains through the animal GI Track (salivary amylases, Acidic pH) , gastric enzymes, bile and pancreatic enzymes and salts etc) Fecal samples from animals administered with the probiotic were checked after 24 hr of dose administration. In order to check the growth and colonization of the animal gut, Fecal samples were taken every fourth day of the feeding chart, i.e., 4^{th} day, 8^{th} day, 12^{th} day, 16^{th} day, 20^{th} day and 24^{th} day. The fecal samples were serially diluted and plated on 200ug/ml rifampicin supplemented MRS agar plates for determining live bacterial CFUs per grams of fecal matter.

3.4.5 *In vivo* adhesion and colonization assay:

To determine the adhesion and colonization ability of the probiotic strains, the animals were sacrificed on the 24^{th} day of dose administration. The animals were administered with general anesthesia followed by cervical dislocation. Approximately 2cm of caecum, small intestine and large intestine was taken and homogenized in PBS. This mixture was serially diluted and plated on 200ug/ml rifampicin supplemented MRS agar plates for determining live bacterial CFU/cm of the sample.

3.5 Preparation of Cell Free Supernatant (CFS)

The probiotic strains were inoculated into 15ml of MRS Broth (de Man Rogosa Sharpe Broth). The media was incubated anaerobically at 37ºCfor 24 hrs. Cultured cells were centrifuged at

10,000 rpm for 10 min. The supernatant was collected and syringe filtered using a 0.2µM syringe filter (Corning, USA). The supernatant was stored at 4ºC.

3.6 Heat inactivated of the cell free supernatant (CFS-H)

Prepared cell free supernatant was heat treated in a Dry heat block (Wealtec Corp, USA) for 10 min at temperature of 90ºC.

3.7 Collection of pathogenic bacteria:

The clinical isolates of two pathogenic bacteria, *Shiga toxin producing E coli* and *Shigella* were obtained from Pakistan Institute of Medical Sciences (PIMS). Both pathogenic bacteria were grown on Salmonella Shigella Agar (SS Agar) (Oxoid Ltd. Basingstoke. Hampshire England). SS Agar was chosen for its selective and differential nature for both, *Shigella* and STEC. It is well recognized for testing clinical specimens and food testing of *Shigella* and *STEC* (Neogen Corporation 2011).

3.8 Antibacterial Activity

3.8.1 Well Diffusion assay

Antibacterial activity of the probiotic strains against Shigella and STEC was determined by agar well diffusion method as described by Kim *et al.,* (2001). Pure colonies of pathogenic bacteria (*Shigella* and *E. coli)* were taken and mixed with 1ml of PBS to achieve 0.5 McFarland turbidity standard. 100ul of this solution was spread on Lb media plates. 100ul of the CFS and CFS-H was added in 8mm wells. MRS was used as a negative control to check the possibility of MRS metabolites contributing to the activity of probiotics. Whereas the Antibiotic Cefotaxime-30ug (CTX-30) (Oxoid Ltd. Basingstoke. Hampshire England) was used as a positive control. The plates were incubated overnight aerobically at 37 C. Zone of inhibition was evaluated by measuring the

diameter of the clear zone. The experiment was repeated four times and appropriate statistics were applied.

3.8.2 Calculation of the Percentage Antibacterial Activity

The percentage activity of the probiotic cell free extracts against *Shigella* and *E. coli* was measured keeping the activity of the positive control (CTX-30) as a standard. The following formula was used:

% Antibacterial Activity = Average zone of inhibition (mm) ÷ Zone of inhibition of antibiotic (mm) × 100

For Statistical Analysis all the graphs were generated using GraphPad Prism.

3.9 Cytotoxic effects of probiotics on cancer cell lines cell lines:

3.9.1 Cell lines

Human cervical cancer cell line (HeLa) were obtained from cell culture bank at Atta-ur -Rahman School of Applied Biosciences, NUST. The cells were maintained at 37˚C with 5% of CO2 flow in a cell culture incubator in 25cm^2 /75cm^2 flasks (Corning, NY, USA) in Dulbecco's Minimal Essential Medium (DMEM) (Sigma Aldrich, USA) with 10% (v/v) of added heat inactivated, sterile and filtered Fetal Bovine serum (FBS) (Biowest, Riverside, US) and 1% (v/v) of antibiotic penicillin and streptomycin (Capricorn, Ebsdorfergrund, Germany).

3.9.2 Sub-culturing of HeLa cells

After the attainment of 80% confluency, the media was discarded and cells were washed by 5ml of Dulbecco's Phosphate Buffer Saline (DPBS) (Biowest, Riverside, USA). 1ml trypsin EDTA (Caisson, Smithfield, US) was added and cells were incubated at 37˚C for 5mins. Cells were resuspended in 5ml of media to deactivate the trypsin and transferred to a 15ml centrifuge tube.

Cell suspension was centrifuged at 1000 rpm for 2mins at 4˚C. The supernatant was discarded and the cell pellet was resuspended in 2ml of complete medium. By readily pipetting up and down cell pellet was mixed to split the aggregates. Cells were added back to the flasks and were incubated at 5% CO_2 at 37˚C.

3.9.3 Cryopreservation

HeLa cells were trypsinized by trypsin EDTA and cell density of 3×10^6 cells/mL. the supernatant was discarded and cells were suspended in a solution containing 9 parts of FBS and 1 part of Dimethyl sulphoxide (DMSO) (CARL Roth, Karlsruhe, Germany). 1ml of suspension was added to a Cryo-vial (Corning, USA). Cryo-vials were placed on ice for 10-15 minutes following this step was the placement of vials at -20˚C for 2hours and finally for long term storage cells were shifted to -80˚C

3.9.4 Cell counting

Cells were trypsinized and centrifugation was done at 1000rpm for 2mins. Supernatant was removed and cell pellet was suspended in 2ml of growth media and was pipetted up and down several times to disperse colonies in order to obtain single cells. 100µl of cell suspension was taken and trypan blue was, such that the ratio of two become 1:1 making the dilution factor 2. The mixture was pipetted up and down gently to allow the through mixing. 10µl of suspension was placed on haemocytometer inlet under the cover slip. Haemocytometer grid was visualized under the inverted microscope TS-100 (Nikon, Japan) cells were counted in five 1mm square grids and Average value of the grids was multiplied with dilution factor and 1 $x10^4$ to determine the number of cells.

3.9.5 Cell viability Assay

1 x10^4 cells of the cell lines were cultured in a 96 well plate (Corning, USA). The plate was incubated in CO_2 cylinder at 37ºC for 24 hours. After 24-hour incubation, culture medium was removed and cell free supernatants with varying concentration along with DMEM were added (Table 3.1) and incubated for 24 and 48 hrs. MRS medium was used as control. After the desired incubation, the cell viability was determined by MTT Assay, 10µl of 5mg/ml MTT Solution was added to each well including controls and plates were incubated for 2 hours. After incubation 100µl DMSO was added to wells and were incubated for 10 mins to allow DMSO to solubilize the formazan crystals. Absorbance was read by ELISA microplate reader at 492nm. The experiment was performed in quadruplicates.

Table 3.1: Varying concentrations of treatments of cell lines.

Experimental Group	
Control Group	MRS is the growth media for probiotics which was used as a negative control.
Blank Group	Only DMEM was added to the wells**.**
CFE treated Group	Cells treated with 200ul/ml, 400ul/ml and 600ul/ml of cell free extract of probiotics.

Cell viability was calculated by the following formula:

% viability= $(A_t-A_b)/ (A_c-A_b) \times 100$

Where by,

A_t= Absorbance value of sample

A_b= Absorbance value of blank

A_c= Absorbance value of control

Percentage cell inhibition was calculated by:

% cell Inhibition= 100 - % cell survival

3.10 DNA Ladder Assay

The cell lines were treated with probiotic cell free supernatant as described previously (write section in which you have mentioned). After the incubation cells were trypsinized and suspended in 400 µl of TE buffer and 14µl of 20% sodium dodecyl sulphate (SDS) solution and 1.5 µl of 20mg/ml of proteinase K solution (company name) followed by overnight incubation at 37˚C. After incubation 500 µl of 6M Nacl solution was added and vortexed for 15 sec. The samples were centrifuged for 20 min at 4,500 rpm. After centrifugation, the upper aqueous phase was collected in a clean appropriately labeled tube. To precipitate the DNA, 500 µl of isopropyl alcohol was added. DNA was precipitated by inverting the tubes several times. This mixture was centrifuged again for 10 minutes at 13,000 rpm and the supernatant was discarded. To the DNA pellet, 200 µl of 70% ethanol was added to wash the DNA and centrifuged for 7 minutes at 13,000 rpm. Ethanol was discarded leaving the DNA stuck to the bottom of the tube. DNA pellet was dried by keeping the microfuge tubes at 37 °C until all the ethanol had evaporated off. To the pellet, 100 µl of TE buffer was finally added and left for 4-5 hours for the DNA to dissolve. The dissolved DNA was loaded on 1% agarose gel. For loading, 6µl of DNA was mixed with 1 µl of loading dye (6x Bromophenol blue). The mixture was then loaded into the wells of the gel carefully and

electrophoresis was performed at 100 volts for 25 minutes in 1X TAE as running buffer. Gel was visualized in UV Transilluminator (Biometra, Goettingen, Germany) and photographed by using gel documentation system (Wealtec Dolphin Doc, Sparks,)

4 RESULTS

4.1 Isolation and Identification of *Bifidobacterium* Species

Three samples were isolated from duck intestine. All the isolates were preliminarily identified as gram positive and catalase negative. Finally, the identification of the isolates was confirmed by Ribotyping (Riboprinter,Dupont USA). Three isolates of Lactobacillus Plantarum were selected and named as strain AA-101, NL-102 & RH-103 respectively.

Figure 4.1 Representative image of bacterial isolates from Duck intestine.

	DuPont ID Similarity	Sample Number	Label	DuPont ID Label	RiboPrint™ Pattern (1 kbp, 5, 10, 15, 50)
1	0.97	631-21-S-1	AR-707	Bifidobacterium animalis	
2	0.94	631-21-S-2	RH-909	Bifidobacterium animalis	
3		631-21-S-3			
4	0.94	631-21-S-4	AA-101	Lactobacillus plantarum	
5	0.87	631-21-S-5	NLD-808	Bifidobacterium animalis	
6		631-21-S-6			
7	0.88	631-21-S-7	NL-102	Lactobacillus plantarum	
8	0.93	631-21-S-8	RH-103	Lactobacillus plantarum	

Figure 4.2: Ribotyping results of the isolated strains.

4.1.1 Gram Staining

Gram stained slides of three isolated strains were seen to retain violet color which clearly indicates that these strains were gram-positive. Figure 4.2 shows rod shaped bacteria which is a characteristic of *Lactobacillus* genus.

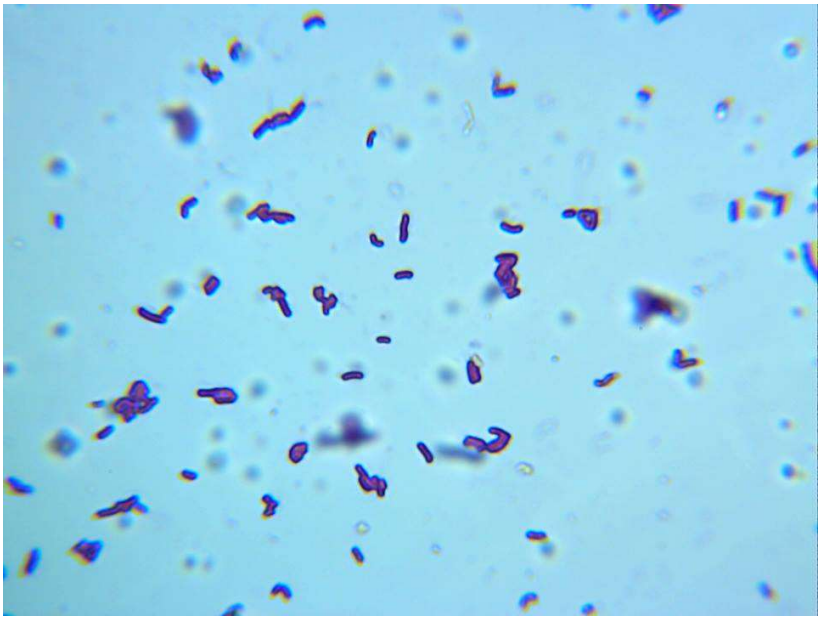

Figure 4.3 Representative image of Gram stained isolate

4.1.2 pH Survival Assay

Survival of the selected bacterial strains was checked in varying Acidic pH range. The selected strains showed tolerance to low pH of 2.5 for at least 30 min.

4.2 *In vivo* Probiotic Potential testing

In order for the isolated strains to be classified as a potential probiotic, their GIT survival, adhesion and colonization of intestinal mucosa was checked. Furthermore, animal models were checked for weight gains, for the entire run of experiment, the animals looked very healthy and no infections or death were reported.

4.2.1 Gastrointestinal Transit

All the strains tested showed strong GIT survival rate as the colonies were detectable in as less as 2 days of probiotic dose. The number of bacteria obtained from faeces of mice on MRS with rifampicin was observed to grow rapidly in the first 4 days. But all the strains did show a significant stationary period of a week, this may explain colonization of certain bacteria (Fig 4.7). Correct the sentence

The faecal samples collected after every four days showed that not only did all the strains survive the gastro-intestinal track environment but were able to grow and thrive in the animal gut which further cements their position as potential probiotic candidates.

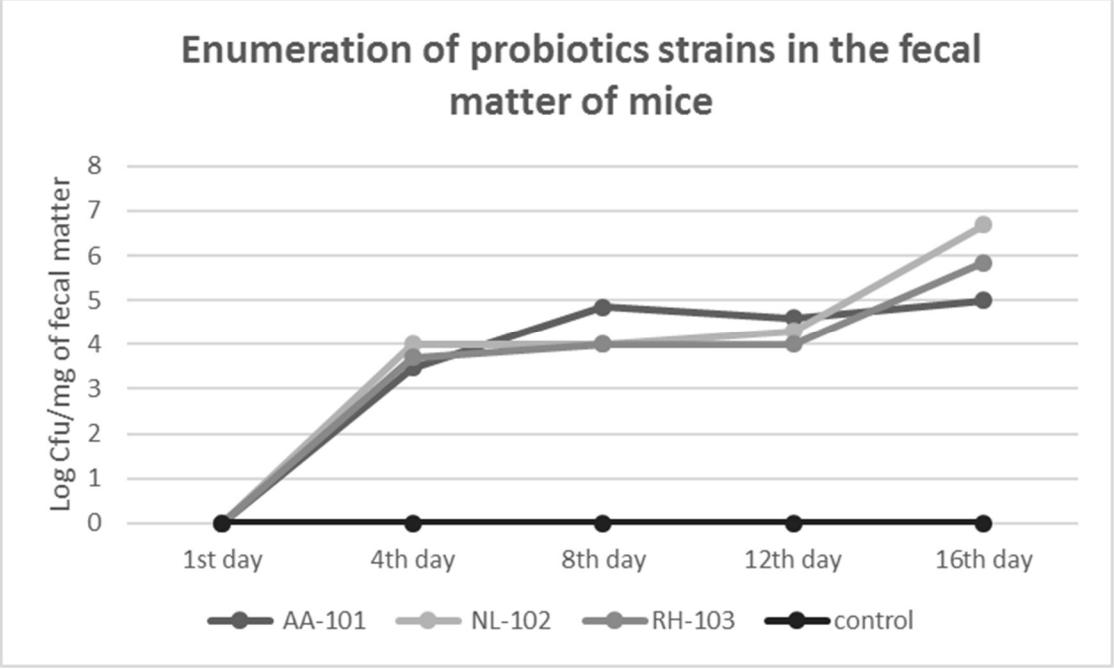

Figure 4.4 Viable cells of *L.plantarum* strains from mice faeces at various days.

After initial stationary phase, from 12thday to 16th day, *L.plantarum* RH-103 and *L.plantarum NLD*-102 bacterial number tends to increase. However, *L.plantarum* AA-101 achieves a stationary

phase late from 8th to 16th day. No resistant strains were detected in the control group for the whole experiment period, which validates the authenticity of the experiment done.

4.2.2 *In vivo* adhesion and colonization assay:

In vivo adhesion and colonization of rifampicin-resistant bacterial strains was monitored by determining the number of microflora in homogenates of small intestine, large intestine and caecum. The colonization preference of all the strains showed a lot of variance. Caecum was observed as a preferable location for colonization, as all the three strains were found to have more than 3 Log CFU/cm^2 of the caecum. While strain NL-102 showed clear preference for caecum, strains AA-101 and RH-103 showed mixed preferences, where former showed preference for small intestine (Fig 4.8)

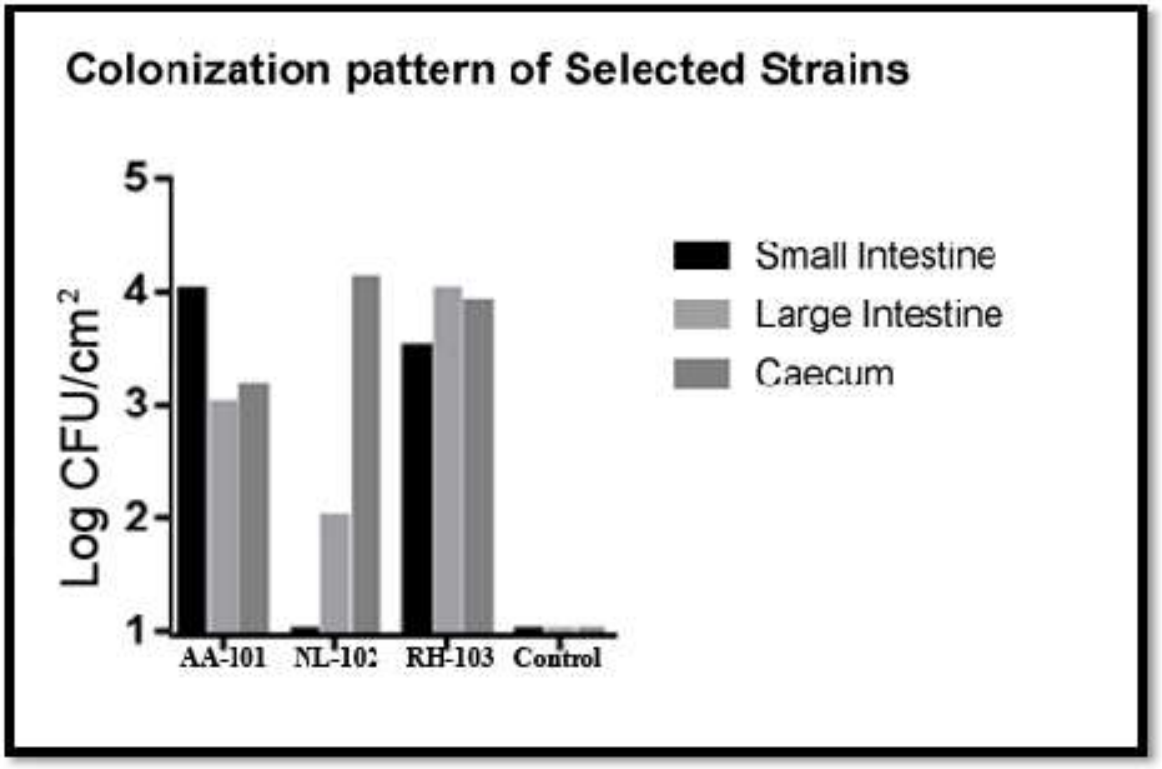

Figure 4.5 Bacterial count in small intestine, large intestine and caecum of mice on 28th day after feeding with *Lactobacillus* strains.

4.2.3 Weight Increase in mice

Weight increase was observed and compared with control, as shown in Figure 4.9 All of the strains showed increased weight gain when compared to control. Strain RH-103 showed considerably higher weight gain as compared to the control, i.e. 58.67% as compared to control (40.1%).

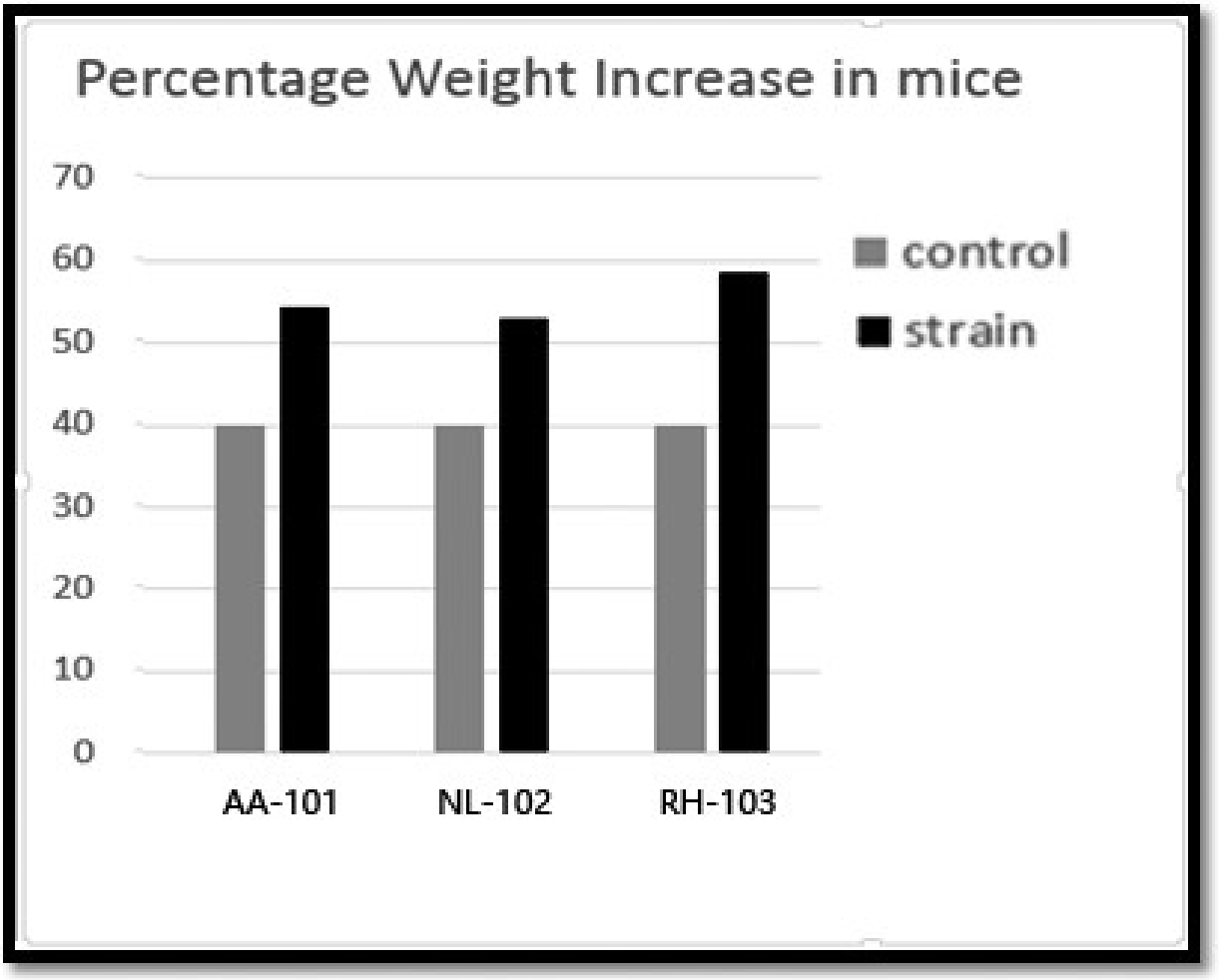

Figure 4.6 The percentage weight increase observed in the mice model in comparison with the control.

4.3 Antibacterial Activity

Strains of *Shigella* and STEC were selected and checked for bactericidal activity of isolated probiotic strains by using well-diffusion method. Antibacterial activity showed that all of *Lactobacillus* isolates had inhibitory activity against *Shigella* and STEC. Maximum diameter of zone of inhibition was found to be 18.6 mm of Cell free Supernatant (CFS) against Shigella, signifying the efficacy of probiotic strains. Antibiotic Cefotaxime-30 µg (CTX-30)(Oxoid,Hampshire, England) served as positive control. No zone of inhibition was observed around the wells treated with MRS (negative control). The result of the antibacterial activity of all cell free extracts against *Shigella* and *E. coli* in the form of average of zone of inhibition (mm) along with their respective pH are shown in Table 4.2.

Table 4.1 Average Zone of inhibition (in mm) given by normal and heat inactivated probiotic cell free supernatants.

Probiotic Strain	***Shigella***		***Escherichia coli***		**pH**
	Cell Free Extracts	***Heat Treated Cell Free Extracts***	***Cell Free Extracts***	***Heat Treated Cell Free Extracts***	
L.Plantarum AA-101	16.8	14.3	18	18	6.53
L.Plantarum NL-102	18.6	16	17.6	17	4.46
L.Plantarum RH-103	13	14.6	16	16	4.46
Cefotaxime-30 (Positive control)	24		27		
MRS (Negative control)	0		0		7

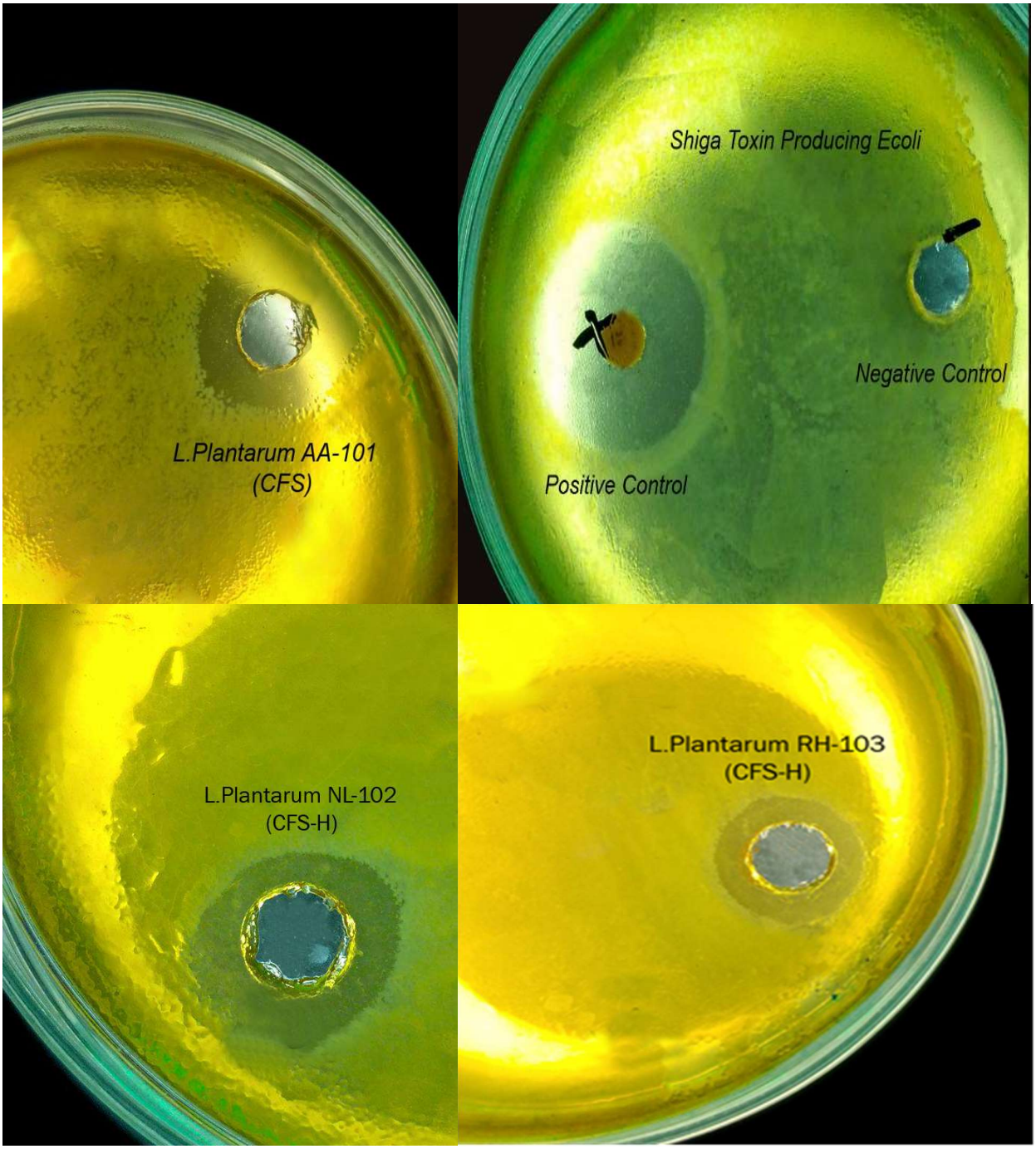

Fig 4.7 Representative images of antibacterial activity against Shigella and STEC. The panel represents (clockwise): activity of CFS- AA-101 against Shigella, positive and negative controinst shigella, CFS-H- RH-103 against STEC, CFS-H- NL-102 against STEC.

4.3.1 Antibacterial activity of Cell free supernatant

All the cell free supernatants (CFS) exhibited a notable inhibitory activity against *Shigella* as well as STEC Fig 4.11. The CFS of *L.plantarum NL-102* showed the highest antibacterial activity against shigella whereas the CFS-H of *L.plantarum AA-101* showed the highest antibacterial activity against STEC, further implying a difference and complexity in mechanism of action and strain specificity of anti-bacterial activity exhibited by these strains.

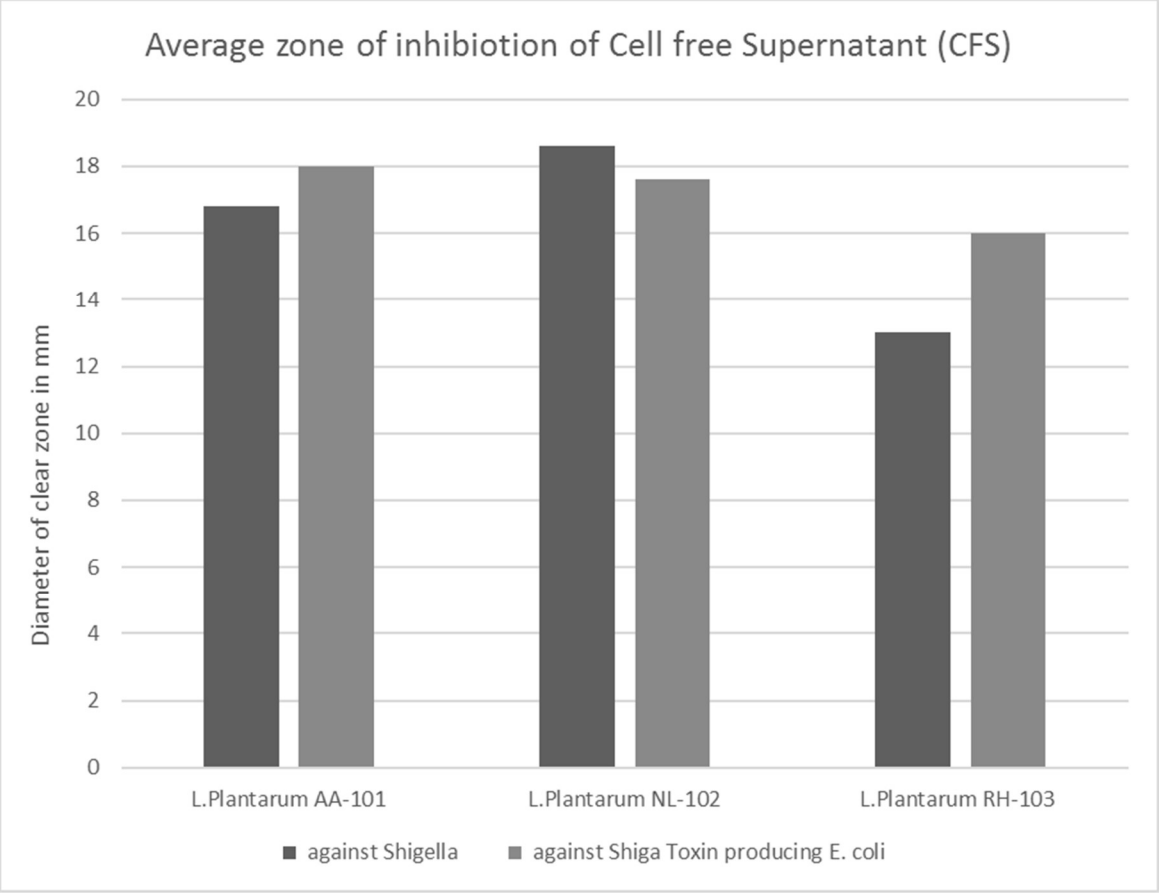

Figure 4.8 Antimicrobial activity of Cell Free Supernatants (CFS) of L.*Plantarum strains* against *Shigella and STEC*

4.3.2 Antibacterial activity of Heat inactivated Cell free supernatant

The heat inactivated Cell free supernatants (CFS-H) exhibited a similar inhibitory activity against *Shigella* as well as STEC as their non-heat treated counterparts (Fig 4.12).The antibacterial activity was retained after heat treatment, implying heat immunity to the constituents responsible.

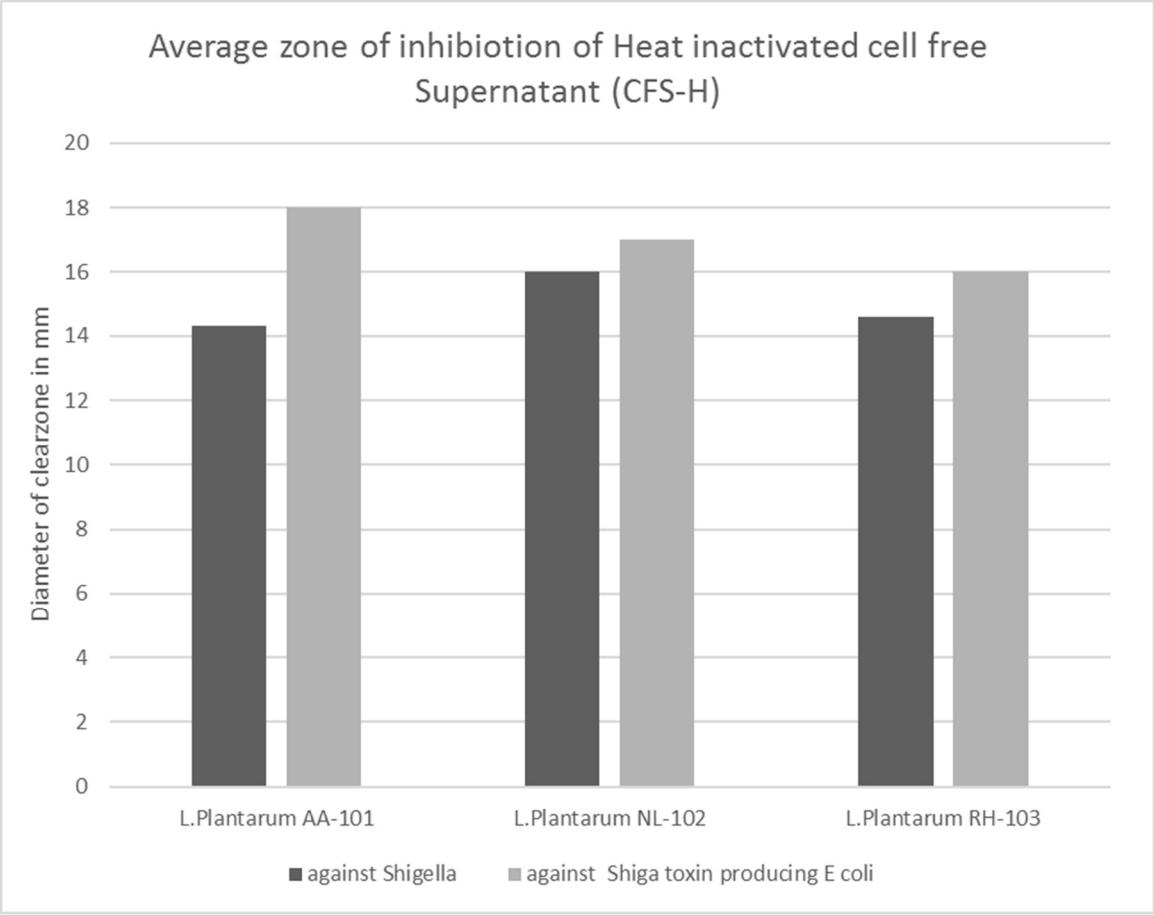

Figure 4.9 Antimicrobial activity of Heat inactivated Cell Free Supernatants(CFS-H) of *L.Plantarum strains* against *Shigella and STEC*.

4.4 Cell cytotoxicity Assay

4.4.1 Treatment for 24 hr

HeLa cell lines treated with cell free supernatant (CFS) of *L.plantarum* strains (200µl/ml, 400µl/ml, and 600µl/ml) showed a significant decrease ($p < 0.05$) in cell viability percentage as compared to control .As shown in Fig 4.8,

L.plantarum AA-101showed the highest cytotoxicity at highest concentration on HeLa, indicating the lowest cell viability. Other strains also showed considerable inhibition in the order of increasing concentrations. The cytotoxicity values corresponding to different concentrations of probiotic extracts indicated that CFS doses inhibited the growth of cancer cell lines in a dose dependent manner.

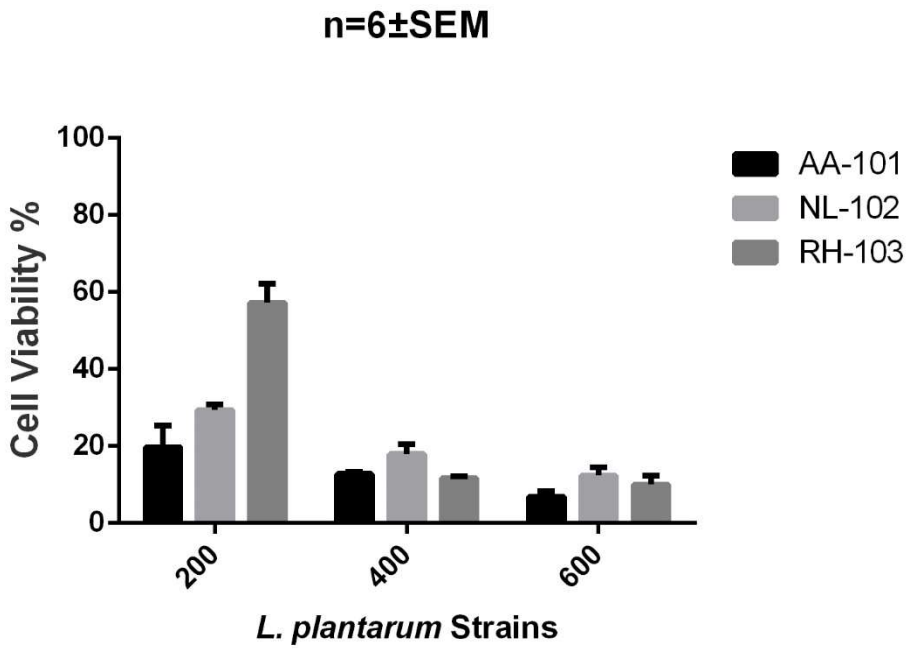

Figure 4.10 The cytotoxic effects of cell free supernatant of bacterial strains (AA-101, NL-102, RH-103) on cancer cell lines for 24 h. All concentrations showed the significant difference at P ≤0.05. Error bars represent standard deviation of each mean.

4.4.2 Treatment for 48 hr

HeLa cell lines treated with cell free supernatant (CFS) of *L.plantarum* strains (200µl/ml, 400µl/ml, and 600µl/ml) showed a significant decrease ($p< 0.05$) in cell viability percentage as compared to controls. Figure 4.9 shows the effect of probiotic dose on HeLa cells, after 48 hrs of treatment. The data indicated that the inhibition occurred in a dose dependent manner. 48 hrs of treatment caused more inhibition of cell growth than 24 hrs of treatment which indicated that the incubation time may affect anti-proliferative activity.

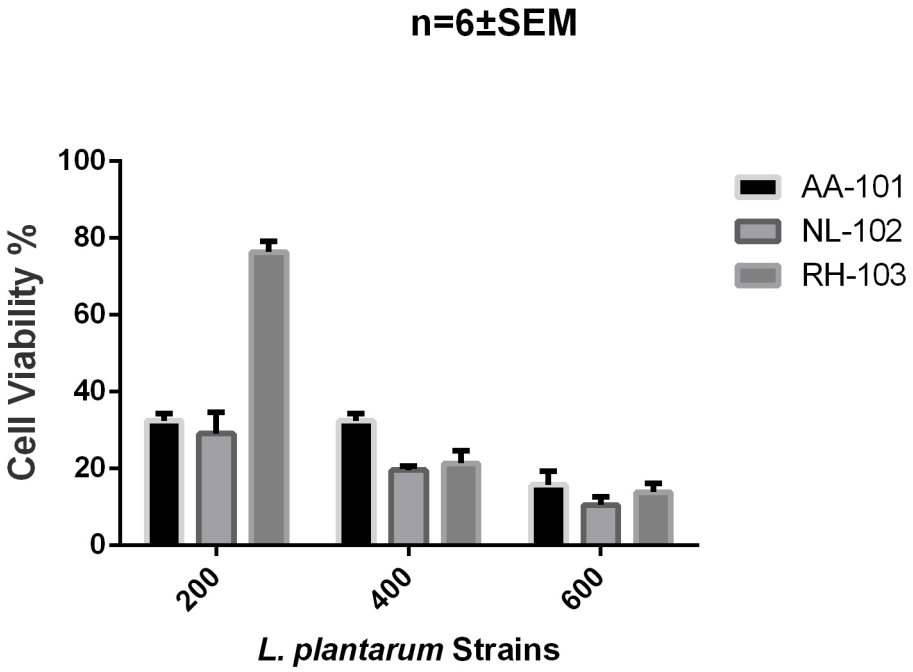

Figure 4.11 The cytotoxic effects of cell free supernatant of bacterial strains (AA-101, , NL-102, RH-103) on human cervical cancer cell line (HeLa) for 48 h. . All concentrations showed the significant difference at P ≤0.05. Error bars represent standard deviation of each mean.

4.5 DNA Ladder Assay

DNA ladder assay was used as a method to assess the morphological changes in cell membrane during cytotoxicity. It was used to visualize apoptosis in treated cells as a ladder pattern of 180-200 bp due to DNA cleavage, which was activated by nuclear endonuclease. We used DNA fragmentation to show the formation of the DNA ladder in gel electrophoresis on Huh-7 and HeLa cell lines as in fig. 4.12.

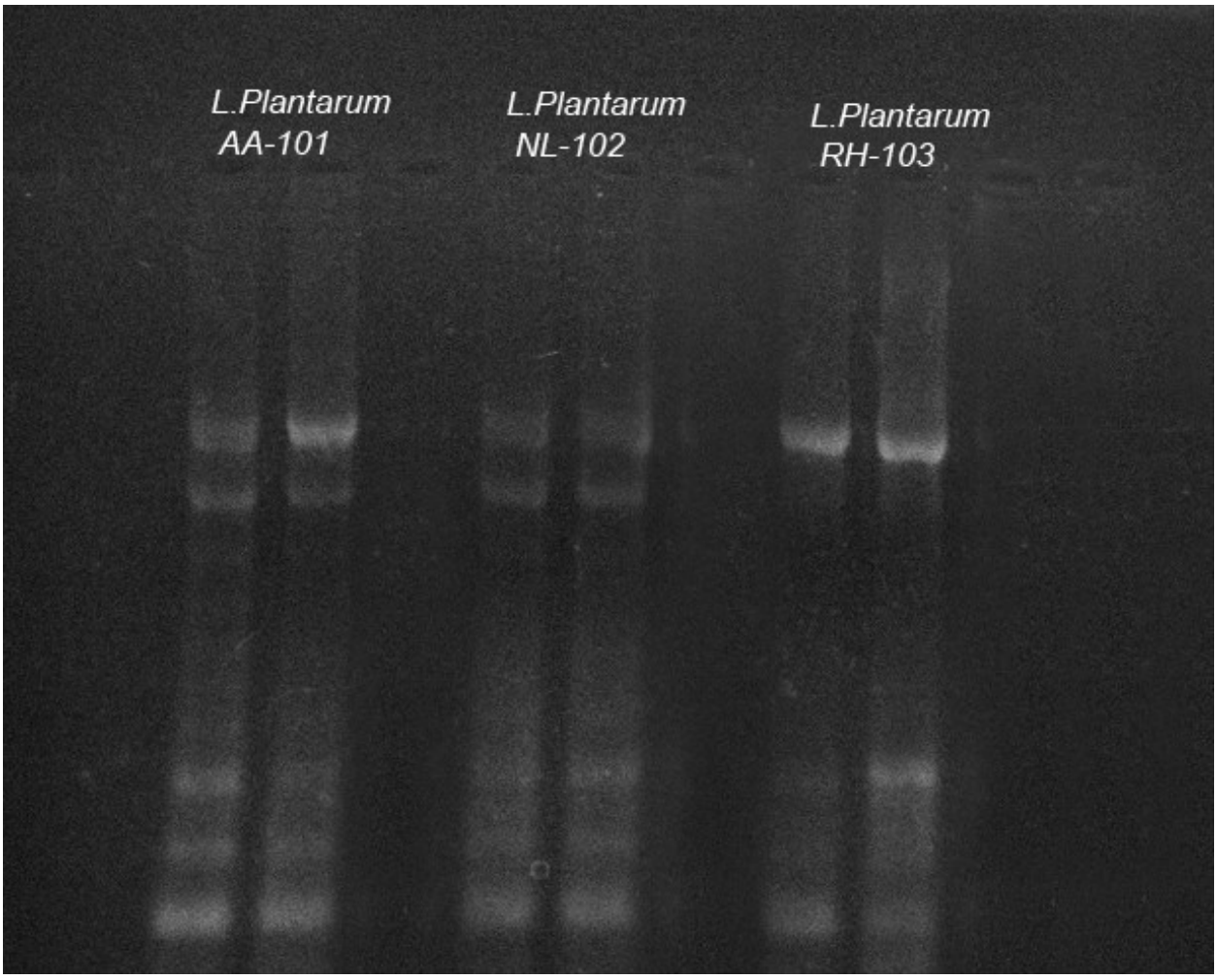

Figure 2.12 Detection of Apoptosis in HeLa Cells. Apoptosis was induced in cells with CFS of probiotic strains.

5 DISCUSSION

For any bacterial isolate to be termed as a potential probiotic, it has to exhibit certain characteristics. These characteristics include, survival in salivary amylase, survival in highly acidic pH, bile salt tolerance and colonization of the GIT in addition to to confer health benefits.

We have chosen the mouse model as an alternative to human GIT, which is much more reliable than *in vitro* systems. By doing this in a single experiment of as less than 2 days, results can be obtained of the survival of the bacterial isolate in the harsh GI environment. As the animals themselves may harbor indigenous micro-flora, this can elaborate additional properties of the strains to compete with the previously establish indigenous flora. However, this also makes the differentiation of our isolate from the background much more difficult.

Rifampicin tagging was used in our experiments for differentiation of probiotic strains from the animal's indigenous microflora. Rifampicin tagging not only is an efficient technique for differentiation of the bacteria but also lasts for all the generations. As rifampicin resistance is not transferable to other bacteria. It proves to be an ideal candidate for this purpose. As evident from the results, all of the tested strains showed strong GIT survival. This is the result of smart isolation techniques. Because the strains were isolated from duck's intestines. Their natural location of prevalence is quite similar to the environment in which they are expected to thrive

Colonization preference of the bacteria is a strain dependent characteristic; different strains of the same specie tend to have different colonization pattern. The preference for a certain location for colonization can be related to different properties. As evident from our results a relationship can be established between the colonization pattern and number of live bacteria in fecal matter. As a "stationary phase" is observed, where bacteria do not increase in number in the fecal matter. This can indicate the colonization and adhesion of the mucosa. As more cells colonize, there number does not increase in the faeces even though a constant daily dose is maintained.

This can be seen in the case of strain NL-102 and strain RH-103 which have shown a colonization preference for caecum. And have shown the prominent stationary phase from 8^{th} to 12^{th} day and then tend to increase afterwards. on the other hand, strain AA-101, the only strain with a clear preference for small intestine has shown no significant changes after 8^{th} day and was observed to remain unchanged. This may be because of highly nourishing caecum environment as compared to the small intestine. This may give the strains having caecum preference an advantage over the small intestine preferring bacteria. Strain RH-103 overall has shown to be most adherent of the given strains, although no preference pattern is visible, it has shown to be among the most adherent in all the locations measured.

For centuries, antibiotics have been used against these infections but the emergence of antibiotic resistant bacteria has developed a need for novel therapeutic and preventive approach (Hempel *et al*., 2012). In our study, three *Lactobacillus* strains had shown antimicrobial activity against STEC and *Shigella*. In the previous studies by *Lin et al*., (2009) and Messaoudi *et al.,* (2005), it has been

reported that that the antibacterial activity of probiotic bacteria is contributed by many factors including lowering of pH, and the release of both bacteriocin and non-bacteriocin compounds. The previous literature also states that the antibacterial property can possibly be a result of proteins such as mucus-binding protein (MUB) and a novel protein (BIF) which are released by the probiotic bacteria (Buck *et al.,* 2005; Hynönen *et al.,* 2002; Fujiwara *et al.,* 2001).

Our results depict that the antibacterial activity continues to exist when probiotics were heat treated, which suggests that the antibacterial agents responsible are stable to heat. This behavior is similar to what was observed by Asahara *et al.,* (2001), who reported that heat killed preparation of probiotic strain, *L. casei* exerts significant antimicrobial activity against *E. coli.* There are no studies yet that have specifically evaluated the heat stability of the bacterial MUB and BIF proteins, therefore, it is unclear whether these proteins are denatured by the heat treatment or are able to retain their activity. It can be concluded from our study that either the proteins do not contribute to the antibacterial activity at all or their mechanism of action is not disturbed by heat treatment. The strains which show fairly heat stable antibacterial activity after heat treatment may have a potential application in the food industry. It implicates that these probiotic extracts can survive the harsher food processing methods.

Another reason of inhibition of STEC and *Shigella* growth is the release of organic acids produced by probiotic strains. Organic acids are acidic in nature and are associated with the lowering of pH (Alakomi *et al.,* 2005). They are natural antimicrobial agents and are widely added in foods as preservatives, they inhibit the growth of food borne pathogens *E. coli* and *Salmonella* (Van *et al.,* 2006). In our experiment we have observed that even though there is a lot of difference in pH of each strain's supernatant, hence in their organic acid content. There activity is quite similar and remains independent of the pH observed. Which indicates that the mechanism of action of such

strains is quite complex and may be made up of different factors Therefore, we conclude that the antibacterial activities are strain specific. Organic acids are not the sole factors in producing the antibacterial effects. This reinforces the conclusions of previously conducted studies that the antibacterial activity of probiotics is contributed by a number of factors.

Preliminary data suggests that probiotics could be used as potential anti-cancer agent. Lactobacilli and Bifidobacterium are the most prominent probiotic bacteria used in testing probiotic activity against cancer cell lines (Wang *et al.,* 2014). Probiotics mainly show their anti-carcinogenic effect by elevation of immune response (Wang *et al.,* 2014). There are several mechanisms proposed for probiotic mediated immunomodulation that include binding and removal of carcinogens, alteration in number and metabolic activities of bacteria that produce putative carcinogens and cancer promoters, and production of anti-tumorigenic compounds in colon (Hirayama & Rafter, 2000).There are isolated reports that indicate that certain LAB strains exhibit anti-tumour activity by producing anti-oxidative enzymes that protects the cell from carcinogen induced damage the enzymes include glutathione-S-transferase, glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase however, no testing is available on human subjects (Er*et al.,* 2015).

In our study, we evaluated the effect of 3 sub species of *Lactobacilus Plantarum* on human liver cancer cell line (HuH-7) and human cervical cancer cell line (HeLa). Cell viability was tested using MTT assay which is the most common assay used to measure metabolic activity of viable cell.

In our experiment all the *L.Plantarum* strains inhibited the growth of both HuH-7 and HeLa cells in a dose dependent manner. As seen in Figure 4.8 and 4.9, the trend shows decreasing viability with the increasing concentration. Antitumor activity of probiotics has been attributed to

upregulation of immune response mainly. But in invitro experiments. This immunomodulation is impossible, which suggests that in addition to this method, probiotics also show anti-tumor activity by direcinduction of apoptosis through unknown mechanisms. The confirmation of apoptosis shows that this involves certain receptor responces and enzyme cascades. It should be kept in mind that strain specific properties exist in probiotics, thus some strains might not show similar activity. Cebeci and Gürakan (2003) explained the strain specificity and proved that some strains are better candidate to be used as probiotics than others. In our study, strain RH-103 gave better inhibitory results as compared to two other strains. Haghshenas *et al.,* studied the effect of Acetobacter strain secreted metabolites on several cancerous cell lines for 12, 24, and 48hrs. His findings suggested that cell viability decreased when the incubation time was increased (Haghshenas *et al.,* 2015), which is in line with our findings.

Through our study, we found out that HeLa cells are more sensitive to the probiotic treatment than HuH-7 cells at 24hours of treatment. The decreasing cell viability with increasing concentration indicates the positive relationship between probiotic dose concentration and cell viability. HeLa cell line at 48 hours of treatment showed greatest inhibition and thus the low cell viability. Probiotics are known for their anti-colorectal cancer prevention and considerable research has been done on this area (De Moreno & Perdigon, 2010). Our work has shown that the isolated strains have the potential to inhibit the proliferation of HuH-7 and HeLa cells. However, the actual mechanism of action still needs to be elucidated.

6 CONCLUSION

From the current study it can be concluded that, all the three strains isolated have a great potential to be used as probiotic. They have shown the ability to survive the harsh GIT environments and colonize in the intestinal mucosa, they have shown to be preliminarily safe to animal models, without any case of translocation or sepsis, but have increased weight gain as a sign of health.

Anti-bacterial activity of the aforementioned strains has also been established against food borne pathogens like Shigella and STEC, there activity has been noted to be independent of organic acid concentration and fairly resistant to heat treatments. It has been observed that their antibacterial activity along with other characteristics is multifactorial and strain specific.

Although a wide range of preliminary studies has been carried out to study the effect of probiotics on cancer cell lines which gave positive results yet the mechanism by which this inhibition occurs is still not clear. Our initial testing suggested that the cell free extract of *Lactobacilus Plantarum* can be used as potential anti-cancer agent against human Liver and cervical cancer cell lines but mechanism of action is not yet illustrated. The multifactorial and strain specific nature of these activities hinder the formulation of trends. A lot of experimentation on a single strain can allow the explanation of its mechanism of action for displaying such activities. Further experimentations like safety testing and shelf life survival are needed in order to fully characterize the mentioned isolates as “Probiotics”.

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