Screening and Genetic Modification of Antimicrobial Protein Producing Probiotic Lactic Acid Bacteria

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Declaration

We declare that the thesis entitled **Screening and Genetic Modification of Antimicrobial Protein Producing Probiotic Lactic Acid Bacteria** have been prepared by us under the guidance of Dr. Sriram Seshadri, Assistant Professor of Institute of Science, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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List of Abbreviations

Abbreviations	**Full name**
Amp^r	Ampicillin resistance
A. hydrophila	*Aeromonas hydrophila*
BCP	Bromocresol purple
Bp	base pairs
Cm^r	Chloramphenicol resistance
Conc.	Concentration
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
g/l	Gram/Litre
GFP	Green fluorescence protein
GI	Gastro- intestinal
GMOs	Genetically modified organisms
HPLC	High performance liquid chromatography
Kb	Kilobase
LAB	Lactic Acid Bacteria
LB	Luria Bertani
M	Molar
Ng	Nanogram
Mg	Miligram
Min	Minutes
mM`	`Millimolar

$mmoll^{-1}$	Milimole /Litre
MRS	deMann, Rogosa, Sharpe
MTCC	Microbial Type culture collection
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RPM	Rotation per minute
S. typhi	*Salmonella* Typhimurium
S. paratyphi	*Salmonella paratyphi*
SDS	Sodium dodecyl sulphate
Sec	Second
S. flexnerii	*Shigella flexnerii*
UPEC	Uropathogenic *E.coli*
TE	Tris EDTA
λ DNA	Lambda DNA

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Abstract

Lactic acid bacteria (LAB) are considered as generally regarded as safe organism (GRAS). Exploitation of Lactic acid bacteria is advantageous not only in improving the microbial safety of food but also as a probiotic in animals and humans to improve the balance of microflora and to inhibit pathogenic bacteria in the intestinal tract. Screening and isolation of such Lactic acid bacteria from different sources, having probiotics characteristics can be of great importance.

In the present study two isolates of lactobacilli, SRN3 and SRN4 from male wistar rat fecal sample were isolated from 36 isolates and screened by various screening methods such as screening in MRS media in presence of Bromo cresol purple dye (BCP) (0.17g/L), MRS+Vancomycin, 2.5 pH adjusted MRS agar plate as well as MRS+0.3% bile (Oxgall) agar plate, sensitivity against various antibiotics and characterization for its probiotic properties such as cell surface hydrophobicity, autoaggregation and coaggregation. Three isolates of lactic acid bacteria, SSR11, SSR14 and SSR16 from fish intestine were isolated from 56 isolates and were screened by various screening methods such as MRS+BCP (0.17g/L), MRS+Vancomycin ,2.5 pH adjusted MRS agar plate as well as MRS+0.3% bile (Oxgall) agar plate, sensitivity against various antibiotics and characterized for its probiotic properties such as acid and bile tolerance, cell surface hydrophobicity, autoaggregation and coaggregation. The antimicrobial activity of the cell free supernatant of all the isolates was determined and found to be effective against different pathogens

The crude protein from all the isolates were obtained by ammonium sulphate precipitation and their molecular mass was determined by Sodium Dodecyl Sulphate Poly Acrylamine Gel Electrophoresis. The antimicrobial activity of SRN4 crude protein was found to be of bacteriostatic mode of action against *A. hydrophila*. Separation and purification of antimicrobial protein of SRN4 was performed by using Superdex 75 (10/300 GL) linked to Reverse Phase-High Performance Liquid Chromatography. The eluted fractions from Size Exclusion Chromatography would be checked for antimicrobial activity and will be purified.

The genetic modification of lactobacilli isolates SRN3 and SRN4 is needed to improve the effectiveness of its existing properties and to add new beneficial activities such as its use as a delivery system. Three different strategies have been applied to add new beneficial properties in both the isolates SRN3 and SRN4. The antimicrobial activity of isolates can be increased by incorporation of ColE2 (colicin) gene through genetic alteration. The shuttle vector pRV86 was used for the incorporation ColE2 gene and eletrotransformed into *Lactobacilli* isolates.

For their application as a delivery vehicle and expression system, incorporation of a heterologous signal peptide *slp*A which provides extracellular secretion properties of the required gene of interest is needed. The secretion vector pSLP111.3 was used to get the *slp*A signal peptide by corresponding restriction endonucleases and will be incorporated into pRV86 and further transformed into probiotic isolates.Tagging of fluorescent labels by green fluorescent protein (*gfp*) to the isolates would be a beneficial approach to investigate the colonization potential of probiotics in the gastrointestinal tract after oral administration.

Summary

A probiotic has been defined as a "live microorganism which when administered in adequate amounts confers a health benefit on the host". The selection criteria for probiotics include lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to gastrointestinal mucosa and competitive exclusion of pathogens.

The present investigation was to isolate Lactic acid bacteria from different sources, characterize for their probiotic properties and genetically modify them for their use in therapeutic applications.

Lactic acid bacteria (LAB) constitutes one of the dominant genera in the mammalian intestine. Mice and rats are often used to test probiotic organisms and microbiota analysis. Hence, it is better to know the lactic acid microbial flora having potential probiotic properties of these laboratory animals. (1).

Two isolates (SRN3 and SRN4) were screened based on their morphological, biochemical characteristics and Polymerase Chain Reaction amplification by *Lactobacillus* specific 16S rRNA primer. These isolates were of *Lactobacillus* species and showed good tolerance against stimulated gastric juice and bile salts. These two lactobacilli isolates SRN3 and SRN4 showed strong antibacterial activities against *Bacillus subtilis,* Uropathogenic *Escherichia coli, Shigela flexneri, Salmonela* Typhimurium and *Salmonela paratyphi.* The isolates SRN3 and SRN4 were found to posses higher cell surface traits such as hydrophobicity, autoaggregation, and coaggregation capacity. All the lactobacilli isolates were susceptible to all the tested antibiotics, except vancomycin.

Indian fish pathologists are looking at probiotics as a potentially useful disease prevention measure in aquatic farms, and active research is continuing in this regard. However, the efficiency of probiotic isolates from tropical freshwater species is less studied and needs further exploration. In this study, probiotic characterization of lactic acid bacteria isolated from the small intestine of tropical freshwater fish was explored. The three LAB isolates SSR11, SSR14 and SSR16 respectively were isolated having antimicrobial activity against *Aeromonas hydrophila, Psudomonas aeruginosa,* UPEC, *S. flexnerii* and *S. Typhimurium.*.

These isolated LAB were sensitive to various antibiotics such as kanamycin, ampicillin, tetracycline, chloramphenicol , vancomycin etc.The morphological study of the isolates by Grams staining proved it to be coccoid in shape. For the identification of strain at molecular level through PCR amplification by specific 16SrRNA primers is under progress.

The crude protein was isolated by ammonium sulphate precipitation from all the LAB isolates and their molecular weight was determined by performing Sodium dodecyl sulphate polyacrylamide gel electrophoresis. The antimicrobial activity of crude protein of SRN4 isolate was checked against *A. hydrophila* and was found to be of bacteriostatic in nature. Purity of crude SRN4 protein was checked by performing Reverse phase liquid chromatography. Thereafter Size exclusion chromatography was performed to purify the antimicrobial protein and 24 fractions were collected and each fraction will be tested against pathogen, the fraction resulting in zone of inhibition will be further purified.

Recently, several recombinant LAB secreting heterologous proteins have been developed which includes *Lactococcus lactis*, *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus brevis* and *Lactobacillus jensenii*. The recombinant LAB is used as live delivery agents for biotherapeutic applications. Through genetic engineering, it is possible to strengthen the effects and create completely new probiotics. Because of widespread industrial and medical importance, there is increased interest in the manipulation and improvement of *Lactobacillus* strains using genetic engineering techniques.

Construction of expression vector consisting of a strong promoter and signal sequence having extracellular secretion can be of great importance. The signal peptide, *slp*A was taken from pSLP111.3 plasmid. The strong constitutive promoter p*ldh* and *gfp* protein were taken from pRV86 plasmid (2). Expression vector pSLP111.3 was digested with restriction enzymes *Nhe*I and *Hind*III to obtained *slp*A signal peptide. The linear fragment of *slp*A was made blunt end with blunting enzyme T4 DNA polymerase by 5'-3' polymerae activity. The pRV86 plasmid was digested with *Sph*I ,was filled with blunting enzyme T4 DNA polymerase by 5'-3' polymerase activity. Both the linear product pRV86-*slp*A will be ligated and transformed in *E.coli* DH5α and confirmed by plasmid isolation and restriction digestion.

ColicinE2 gene coding for colicin was amplified by polymerase chain reaction and ligated to previously blunt ended pRV86 vector by using T4 DNA polymerase 3'-5' exonuclease activity. Incorporation of colE2 gene will enhance the antimicrobial activity of isolate against pathogenic *E. coli.*

To monitor the colonization of the isolates, pRV85 vector having GFP was electroporated in SRN3 isolate. As the transformation results are awaited, the transformation and colony phenotype will be confirmed by plasmid isolation and PCR, and secretion potential by protein expression.

1. INTRODUCTION

A probiotic has been defined as a "live microorganism which when administered in adequate amounts confers a health benefit on the host". The bacterial genera which are most commonly used in probiotic preparations include: *Lactobacillus, Bifidobacterium, Escherichia, Enterococcus, Bacillus and Streptococcus.* The selection criteria for probiotics include lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens. The management of gastrointestinal infections caused by pathogenic microorganisms is the most extensive studied field of probiotics. The development of alternative therapies based on bacterial replacement is considered important due to the rapid emergence of antibiotic resistant pathogenic strains and the adverse effects of antibiotic therapies on the protective microbiota.

Probiotics have also exhibited antagonistic effects against pathogens belonging to the genera *Listeria, Clostridium, Salmonella, Shigella, Escherichia, Helicobacter, Campylobacter* and *Candida* .The possible mechanisms underlying these antagonistic effects include competition for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation, and immune stimulation. (3).

The aggregation is a desirable property for probiotics to achieve an adequate mass in order to manifest beneficial effects. The ability to coaggregate with other bacteria such as pathogens may have an advantage over non-coaggregating organisms, which are more easily removed from the intestinal environment. Adherence of bacteria to intestinal epithelium is prerequisite for colonization. Adhesion is a complex process involving non-specific (hydrophobicity) and specific ligand-receptor mechanisms. Adherence of bacterial cells is usually related to cell surface characteristics. Many authors have reported that the coaggregation abilities of *Lactobacillus* species might enable it to form a barrier that prevents colonization by pathogenic bacteria. (3)

The cells aggregate due to the interaction of cell surface components such as lipoteichoic acid, proteins and carbohydrates as well as soluble proteins. Studies on the mechanism of autoaggregation in lactobacilli showed that proteins present in the culture supernatant and proteins or lipoproteins located on the cell surface are responsible for cell aggregation.

Furthermore, it was observed that spent culture supernatants of autoaggregating lactobacilli mediate not only the autoaggregation but also aggregation of lactic acid bacteria and even *E. coli* (4).

It is better to know the lactobacilli flora with potential probiotic properties of laboratory animals such as mice and rat as they constitute the dominant genera in their large intestine (1).

Indian fish pathologists are looking at probiotic isolates from tropical freshwater species as a potentially useful disease prevention measure in aquatic farms, and active research is continuing in this regard. The efficiency of probiotic isolates from tropical freshwater species is less studied and needs further exploration. According to (5), there is no report on the probiotic efficiency of intestinal microbiota of tropical freshwater fish. In this study, probiotic characterization of lactic acid bacteria isolated from the small intestine of tropical freshwater fish was explored.

In the present study the attempt was made to isolate Lactic acid bacteria from rat fecal sample and from small intestine of tropical fresh water fish. For probiotic characterization, hydrophobicity, co-aggregation properties with selected enteric pathogens and autoaggregation properties were investigated.

Generally, lactic acid bacteria (LAB) are regarded as safe and useful commensal bacteria, which are known as probiotics and starters for food fermentation. Recently, several recombinant LAB secreting heterologous proteins have been developed which includes *Lactococcus lactis*, *Lactobacillus plantarum* and a strain of *Lactobacillus jensenii*. The recombinant LAB is used as live delivery agents for biotheraputic applications. The, *L. lactis* was genetically modified for the treatment of inflammatory bowel diseases in a murine model. The *L. lactis* secretes active interleukin 10 (IL-10). The successful study thereafter progressed to a clinical trial in humans. Delivery of anti-infective by LAB is also under investigation. Secretion of human CD4 in a strain of *L. jensenii* and secretion of microbiocidal cyanovirin-N in *L. lactis* and *L. plantarum* have been explored for the prevention of HIV-1infection.These studies suggested that protein-secreting systems in

LAB could be useful and offer a promising strategy for medical applications in the future (6).

For this purpose, construction of a plasmid with a expression cassette consisting of a strong promoter, a signal sequence, and a heterologous model protein is needed.

Various studies have been reported using *Ldh*, a very strong constitutive promoter (7,8). The activity of this promoter is only marginally affected by energy source used to grow the bacteria (7). *Ldh* from *L. agilis* 3 is strongest constitutive promoter among other constitutive promoters like p*slpA*, p23, p144, *pcy*sk, *ppg*m and *phl*b (9). The ldh was successfully used to express the gfp gene in *L. reuteri* strains (10).

In bacteria, protein targeting is accomplished via protein sequences or motif called signal peptides. Signal peptide(SP) (sometimes referred to as signal sequence, leader sequence or leader peptide, is a short (5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretary pathway (11). Signal peptide *slpA* subunits can be modified to carry heterologous protein as a uniform recombinant S layer on the *Lactobacillus* sp. cell surface (12) and opens up the possibility for its use as an antigen carrying vector (13). S-layer proteins are very efficiently expressed and the expression and secretion signals of *slp*A have been used in heterologous protein production systems (14).

An *E. coli-Lactobacillus* shuttle vector plasmid, pRV86, having p*ldh* a strong constitutive promoter and gfp protein, established by (2), was used as the base plasmid. The signal peptide, *slp*A was taken from pSLP111.3 plasmid. To increase the antimicrobial activity of *Lactobacilli*, colicin gene ColE2 derived from pColE2-P9 vector was cloned in pRV86.

In the course of the study, this modified shuttle *Lactobacillus–E. coli* vector would be electroporated into lab isolated *Lactobacillus* strains and protein expression study would be carried out.

To investigate the colonization potential the isolate SRN3, the isolate was genetically modified. The green fluorescent protein (*gfp*) was used to monitor colonization of this isolate by electroporating pRV85 plasmid in it.

2. REVIEW OF LITERATURE

In recent years, the consumer has been increasingly confronted with functional food products which are claimed to promote his /her health and well-being. At the centre of these food products are the so-called pro- and prebiotics (15).

The term ‘probiotic’ (Greek: ‘for life’) originally referred to a phenomenon observed when two organisms were grown together, in which substances produced by one organism stimulated the growth of the other organism. These substances were referred to as

‘probiotics’. This term was subsequently used to describe living preparations of microbial cells that could be administered to animal and humans to promote the health of its consumer (16).

Fuller (1991) defined probiotics as ‘a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance’(17). This definition was broadened by Reuter (1997) to ‘any viable mono- or mixed culture of microorganisms which beneficially affects the host on the indigenous microflora’(18). Klaenhammer and Kullen (1999) compiled selection criteria for probiotic strains, which include:

(1) Appropriateness (accurate taxonomic identification, normal inhabitant of the host species targeted, nonpathogenic, nontoxic, GRAS status).

(2) Technological suitability (amenable to mass production and storage: sufficient growth, recovery, concentration, freezing, storage, dehydration and distribution; viability at high numbers; stability of desired characteristics during culture preparation, storage, and delivery); provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes; genetic stability.

(3) Survival competitiveness and establishment (suitable of survival, metabolic activity, and proliferation at the target site *in vivo*, resistant to acid, resistant to bile, able to compete with normal microflora, including the same or closely related species: potentially resistant to acid, bacteriocins and other antimicrobials produced by the residing microflora, and adherence and colonization potential preferred).

(4) Performance and functionality (able to exert one or more clinically documented health benefits, antagonistic towards pathogenic/procarcinogenic bacteria, production of antimicrobial substances such as bacteriocins, immunostimulatory, antimutagenic and anticarcinogenic activity, as well as production of bioactive compounds such as enzymes, vaccines or peptides). Because of the potential health benefit of probiotic bacteria, these organisms are increasingly being incorporated into dairy and other foods. Claimed health benefits include prevention or alleviation of diarrhoea, antimicrobial, anticarcinogenic and antimutagenic properties, improvement of lactose tolerance and reduction in serum cholesterol levels (19,20).

Dunne et al., (1999) defined criteria by which a microorganism may be considered as a probiotic. Thereby, a probiotic microorganism should:

- Demonstrate non-pathogenic behavior.
- Exhibit resistance to technological production processes.
- Prove resistance to gastric acid and bile.
- Adhere to gut epithelial tissue.
- Be able to persist, albeit for short time, in the gastrointestinal tract.
- Produce antimicrobial substances.
- Modulate immune responses.
- Have the ability to influence metabolic activities (e.g. cholesterol assimilation, vitamin production, lactase activity).(21)

After the respiratory tract, the Gastrointestinal (GI) tract constitutes the second largest body surface area. During a normal lifetime, about 60 tons of food passes by this canal. It is estimated to contain about 100 trillion live bacteria. This is roughly 10 times the total number of cells in the human body. These viable bacteria account, roughly 2-3 lbs of a body's weight and are known as intestinal or gut flora. Normal microflora of GI tract includes *Bacteroides* sp., *Lactobacillus, Enterococcus, Escherichia coli, Bifidobacteria, Clostridium, Eubacterium,* and *Ruminococcus.* Approximately 10^{14} bacteria (10^{11}cells per gram of faeces) estimated to be present in the gut microbiota of an adult individual (22; Fig.1). Fungi, viruses and protozoa may also be present, but these normally form only a minor part of the whole resident population of microorganisms in healthy individuals. The

composition of the gastrointestinal flora differs among individuals, and also during life within the same individual. Several factors, such as diet, aging, climate, illness, infection, medication (especially antibiotics), stress, pH, geographic location, race, socioeconomic circumstances and lifestyle can upset this balance. Intestinal microflora profoundly influences physiological, nutritional, and protective processes. For most favorable "gut flora balance," the beneficial bacteria, like lactobacilli and Bifidobacteria, should predominate, presenting an obstacle to invading organisms. The greater part of the intestinal microflora in a healthy person should be good bacteria. The intestinal microflora provides protection against a wide range of pathogens and yeasts like *Candida albicans.* The greater the imbalance of commensals, the greater is the symptoms. The use of probiotics may be the most natural, safe and common sense approach for maintaining the balance of the intestinal ecosystem.

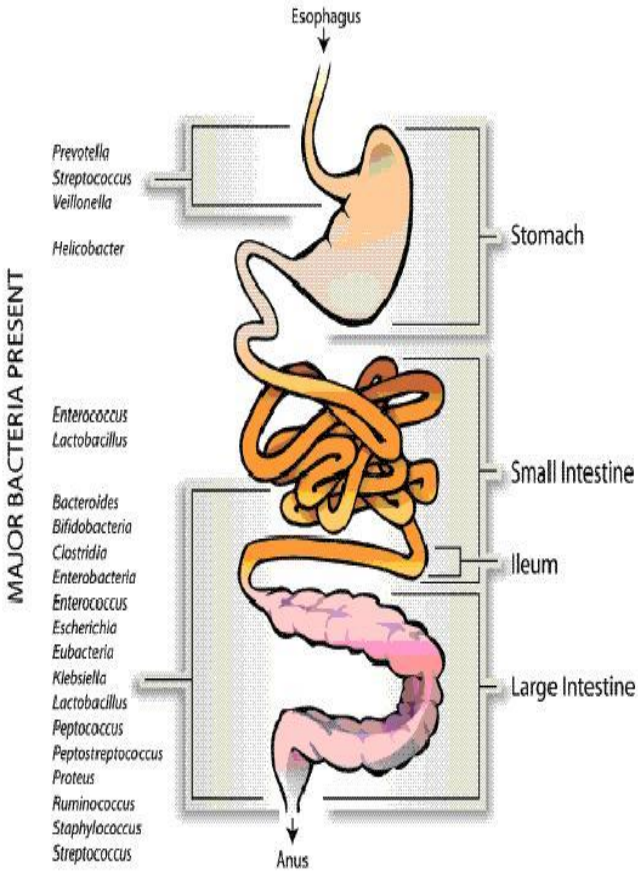


Figure 1: Distribution of nonpathogenic microorganisms in healthy humans (23)

2.1. Mode of action of probiotics:

2.1.1. Mechanism

Schematic diagram illustrating potential or known mechanisms whereby probiotic bacteria might impact on the microbiota. These mechanisms include (1) competition for dietary ingredients as growth substrates, (2) bioconversion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, (4) direct antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colonization and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC: epithelial cells, DC: dendritic cells, T:T-cells (Figure 2).

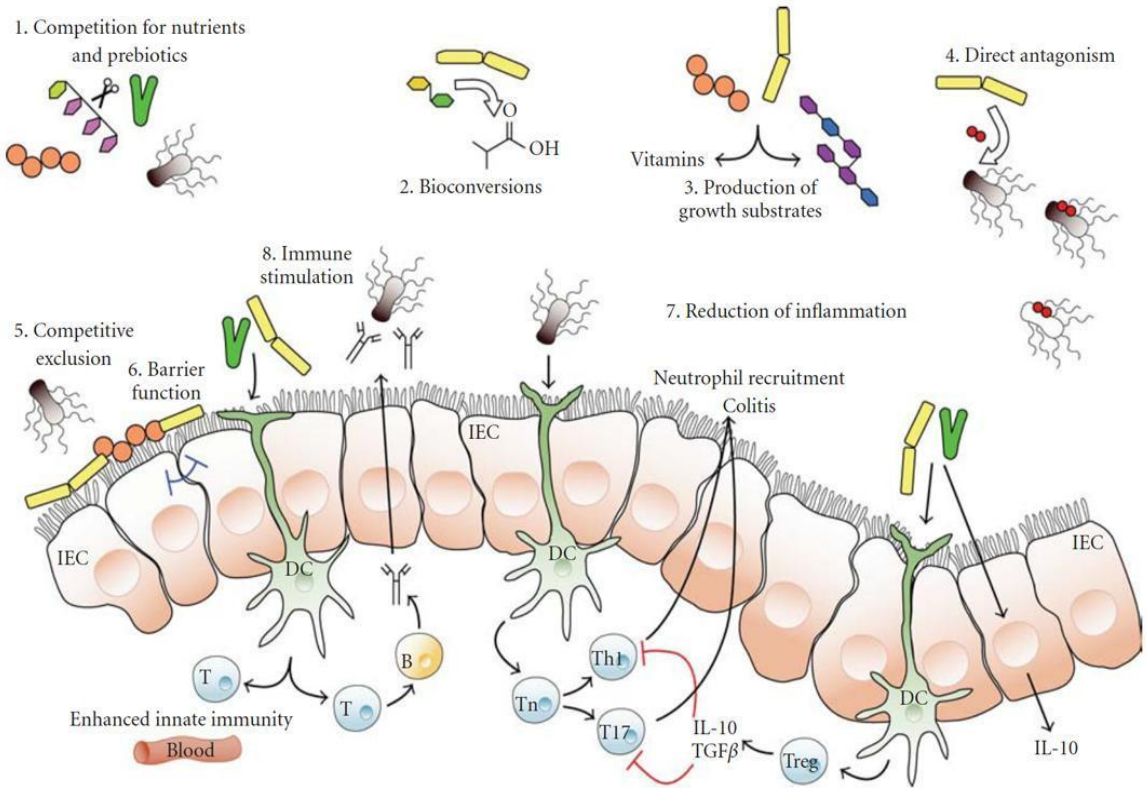


Figure 2: Mechanisms of probiotic bacteria upon the microbiota other bacteria. (www.customprobiotics.com)

Table 1: Therapeutic properties of probiotics and their possible causes and mechenisms (20)

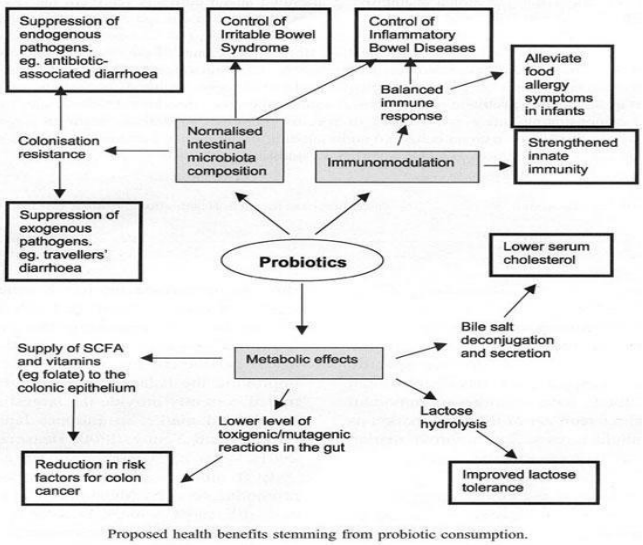
Therapeutic (probiotic) properties	**Possible causes and mechanisms**
Colonization of gut and inhibition of pathogenic microorganisms	Survive gastric acid, resist lysozyme, tolerate high bile salt concentration, adhere to intestinal surface and production on inhibitory compounds i.e. acids, H_2O_2
Improved digestibility of food and enhanced growth of host	Partial breakdown of protein, fat, carbohydrate and improved bioavailability of nutrients.
Alleviation of lactose intolerance	Reduced lactose in the product and further availability on bacterial lactase enzymes for lactose hydrolysis.
Hypocholesterolaemic effect	Production of inhibitors of cholesterol synthesis, deconjugation of bile salt, assimilation of cholesterol.
Anticarcinogenic effect	Inhibition of carcinogens and enzymes involved in converting procarcinogens to carcinogens, inhibition of growth of putrefying organisms and stimulation of host immune system.
Stimulation of the host immunological system	Enhancement of macrophage formation, stimulation of T supressor cells and production of interferon.
Increased vitamin availability to host	Synthesis of group B (folate) vitamins.

Antibiotics have been used as prophylactic and therapeutic treatments to prevent a variety of bacterial infections in human and livestock for more than 50 years. The reduction of antibiotic application in livestock can only be achieved if alternative antimicrobial strategies are accessible. Among those strategies that have been investigated and applied are: diet modification, immunization, feed additives, sanitation, and probiotic bacteria (24,25). A variety of probiotic bacteria have been tested to control animal and food borne pathogenic bacteria in livestock, but in many of them the beneficial effects have not been fully elucidated (17; Table 1).

Many probiotic strains inhibit pathogens by some of the common mechanisms that are by hydrogen peroxide production, acid production, and bacteriocin production or by adhesion or coaggregation with pathogens.

Among all these mechanism bacteriocins are more important. Because compared to antibiotics, most bacteriocins are relatively specific and can only affect a limited number of bacterial species. The specificity of bacteriocins can be particularly advantageous for applications in which a single bacterial strain or species is targeted without disrupting other microbial populations.

2.2. Health benefits of probiotics:



Proposed health benefits stemming from probiotic consumption.

Figure 3: Proposed health benefits stemming from probiotic consumption (27)
Lactic acid bacteria have been extensively studied for its beneficial applications as probiotics.

2.3. Lactic acid bacteria (LAB)

LAB are described as Gram positive, nonsporing, non respiring cocci or rods, which produce lactic acid as a major end product during fermentation of carbohydrates (28). These are catalase negative, non spore forming with coccoid, coccobacilli or rod shaped morphology. They have less than 55% G+C content in the DNA and therefore belong to the *Clostridium* branch of Gram-positive bacteria (29).

Historically, LAB has long been known to be involved in the production of fermented foods. Presently, these products constitute one-quarter of our diet and are characterized by a harmless and safe history, certain advantageous health effects, and prolonged shelf life when compared to non-fermented, fresh foods (30).

LAB are generally considered as 'food grade' organisms. It is assumed that most representatives of this group do not pose any health risks to healthy humans. Most LAB are designated as 'GRAS' microorganisms in the USA based on a long history of safe application in foods. However, some species may act as opportunistic pathogens in rare cases (31,32,33).

Lactic acid bacteria (LAB) are commonly found in many foods such as meat, vegetable and milk, sometimes as dominating microflora (34). Exploitation of LAB as a preservative agent is advantageous not only in improving the microbial safety of food but also as a probiotic in animals and humans to improve the balance of microflora and to inhibit pathogenic bacteria in intestinal tract (35). Intestinal lactic acid bacteria (LAB) for humans are closely associated with the host's health because LAB are an important biodefense factor in preventing colonization and subsequent proliferation of pathogenic bacteria in the intestine (36,37) The currently recognized genera of LAB are *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Streptococcus*, *Dolosigranulum*,

Globicatella, *Lactosphaera*, *Melissioccus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Vagococcus*, *Tetragenococcus*, and *Weissella* (28). Some species of LAB have been claimed as probiotics, such as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus reuteri, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus fermentum, Lactobacillus plantarum,* and *Bifidobacterium species*. To be considered as probiotics, these bacteria should become a part of the normal microbial flora in the intestine, able to adhere and colonize to the intestinal epithelial cells and survive the gastrointestinal passage (38). The gastrointestinal tract of a healthy human is a harsh environment because it contains gastric juices, digestive enzymes, and bile acids. These conditions impose a significant threat to probiotic strains. In addition, low surface tension and immune response also affect the survival of probiotic strains (39). Since one hundred trillion individual bacteria of 100 different varieties inhabit the intestine (40), it is challenging for probiotic strains to become established as gastrointestinal microflora. Thus, organisms that can produce a product that will inhibit the growth or kill pathogenic organisms in the intestinal milieu have a distinct advantage (36)

LAB can exert the following beneficial effects: (a) production of lactic acid and minor amounts of acetic and formic acids, which cause a drop in pH and thereby inhibit the growth of food spoilage or food poisoning bacteria, (b) detoxification by degradation of noxious compounds of plant origin such as cyanogens, (c) production of antimicrobial compounds (e.g. bacteriocins, fatty acids, hydrogen peroxide) which can also inhibit spoilage or food poisoning bacteria, and (d) probiotic effects as of LAB administered as live organisms in food (30).

The present study is focused on screening of antimicrobial protein producing LAB isolates from rat fecal sample and fish intestine and characterization of their probiotic potentials.

2.3.1. *Lactobacillus* as probiotic

Lactobacillus, also called Döderlein's bacillus, is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria (41). They are a major part of the lactic acid bacterial group, named as because many of its members convert lactose and other sugars to lactic acid.

Most Gram-negative bacteria are intrinsically resistant to vancomycin because their outer membrane is impermeable to large glycopeptide molecules (42). Few gram-positive bacteria and *Lactobacillus* species are also intrinsically resistant to vancomycin (43).

Lactobacillus constitutes extremely important group of probiotic bacteria in the gastrointestinal tract of humans (19). In human stomach, the number of lactobacilli is low (< 10^5 CFU/mL). The number of bacteria increases from >10^4 CFU/mL in duodenum to 10^8-10^9 CFU/mL in ileum. lactobacilli can be found in faeces in number ranging from 0 to 10^9 CFU/g (44). *Lactobacillus* species are main constituents of probiotics that fed with the aim of enhancing the immune system, increasing body weight, and improving feed conversion efficiency (45). Moreover *Lactobacillus* improves lactose digestion, reduce gastrointestinal disorders, enhance cellular immunity, and protect against colon cancer (46). They also improve disturbances of the indigenous microflora, upgrade the development of microflora and have antidiabetic and anti-hyperlipidemic effects (47)

Lactobacilli constitute one of the dominant genera in the rat large intestine along with bacteroides, fusiforms, eubacteria, curved rods, and anaerobic Gram-positive cocci (48). It has been reported that certain strains of lactobacilli are beneficial for human as well as in rats for its anti-inflammatory, anti-allergic and reduced side effects of nonsteroidal anti-inflammatory drugs (49). The characteristics of lactobacilli of various origins, including those isolated from rat intestine have been studied and many of them possess properties that make them potential probiotic (50,51,52,53,54). Mice and rats are often used to test probiotic lactobacilli and microbiota analysis.

The *Lactobacillus* strains have been the most extensively studied regarding their functional properties as probiotics. The other studied strains are *Bifidobacterium*, *Escherichia*, *Enterococcus, Bacillus and Streptococcus.*

The objective of this study is characterization of lactic acid bacteria having potential probiotic activity from the gut of healthy rohu (Labeo rohita), a tropical freshwater fish.

With the rapid developments in aquaculture, fish and shrimp farming industries are constantly under threat and severe economic losses due to the outbreak of infectious diseases (55). Members of the genus *Vibrio* and *Aeromonas* have been described as being among the most common pathogenic species in shrimp and fish which causes serious losses in larval and growout phases throughout the world. Use of antibiotics to control these agents has led to problems of drug resistance, brings important changes in the microbiota of the aquaculture systems and surrounding environment and resulted in trade restrictions in export markets (56). Therefore, it is important to seek and combat these pathogens with the development of alternative methods.

As an alternative strategy to these antimicrobial compounds, the preventive use of beneficial bacteria (probiotics) has emerged to improve health and zootechnical performances like survival, production, feed conversion and growth rates of cultured aquatic species (57).

In vitro studies using the agar spot method (58) have shown that *Enterococcus. faecium* (strain IMB 52) has inhibition properties against a wide spectrum of aquatic pathogens including *Vibrio harveyi*, *Yersinia ruckeri*, *Streptococcus agalactiae* and *Aeromonas veronii*. Similar finding was made by Swain *et al.*, (2009) who proved the inhibitory activity of *E. faecium* isolated from brackishwater fish against *V. harveyi* and *V. parahaemolyticus*. This provides the potential applications of *E. faecium* from fish intestine for controlling pathogenic vibriosis in shrimp culture(59). Maintaining the balance of critical parameters and effective disease control remain fundamental requirements for successful aquaculture. In order to withstand the high stocking densities in shrimp and fish production probiotics are a promising feed additive to stimulate animal growth and advance disease resistance. *E. faecium* as probiotic strain in aquatic species is increasingly recognized as safe and can be applied in different combinations. *E. faecium* can grow at a wide range of temperatures and thus has an advantage over other bacteria considering that fish and shrimp are reared at different temperatures and conditions.

In vitro studies and performance trials the potential benefits of *E. faecium* as probiotic feed additive for fish and shrimp, either by stimulating the development of a healthy gut

microflora or by inhibiting pathogenic bacteria like *Vibrio* spp., *Yersinia* spp. and *Aeromonas* spp.

2.4. Genetic Modification of Probiotic Bacteria

Genetic modification of probiotic bacteria is needed to improve the effectiveness of its existing properties (e.g. bacteriocin levels) or to add new beneficial activities (e.g. vaccine presentation). Genetic analysis and manipulation of these bacteria can be important in understanding their probiotic roles and optimizing their performance in vitro and in vivo. The discovery of broad host range plasmids and increasing development of electroporation procedures for DNA transformation results in genetic analysis and modification of lactobacilli (19).

Heterologous gene expression allows construction of novel lactobacilli with potentially valuable characteristics. It has developed a critical need to investigate and control excretion and secretion processes in order to export proteins, enzymes, and potentially antigenic epitopes in lactobacilli. The level of gene expression varies depending upon the gene, promoter, signal sequence and expression host. Therefore to obtain controlled gene expression under gastrointestinal environment, the isolation of strong, weak, regulated or constitutive promoters along with the signal peptides from intestinal lactobacilli is needed. The S-layer genes derived from *L. brevis* (60) and *L. acidophilus* (61) have been cloned and sequenced, and the regulatory and secretion signal peptides of the *L. brevis* S-layer gene has been used in the construction of highly efficient synthesis and export system for heterologous proteins and epitopes.

When using similar expression systems in different *Lactobacillus* strains it is important to be aware that promoters can have different activity levels, depending on the strain in which they are used (62), and that replication efficiencies and plasmid copy numbers can differ. In addition, codon usage of heterologous genes is often different from the expression host, which has been shown to cause reduced expression levels in *Escherichia coli* (63).

Lactobacillus strains show different preferences for codon usage (13) and this could influence the efficiency of translation of a specific antigen.

The mode of action, stability and survival capacity of these genetically modified probiotic strains in the host system can be monitored by incorporating marker gene in the plasmid vector. Stable marker systems with an easily detectable phenotype provide an ideal and important strategy to detect microorganisms in complex environments such as gastro intestinal tract. A few marker genes have been used such as antibiotic resistance genes, lacZ or lux (65,66). GFP, unlike other markers can be detected directly on complex matrices without addition of chromogenic or specific substrates and does not require prior extraction or plating of bacteria (2).

Potential targets for genetic modification and improvement of probiotic strains include: Oral vaccine development and immunostimulation, bacteriocin and other antimicrobial peptides, delivery and production of digestive enzymes, vitamin synthesis and production, adhesions and colonisation determinants, and metabolic engineering to alter products (e.g. polysaccharides; organic acids) or link cultures with specialty prebiotics designed to enhance the performance of a probiotic *in vivo*.

In bacteria, protein targeting is accomplished via protein sequences or motif called signal peptides. Signal peptide (SP) sometimes referred to as signal sequence, leader sequence or leader peptide, is a short (5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretary pathway (11). Signal peptide *slp*A subunits can be modified to carry heterologous protein as a uniform recombinant S layer on the *Lactobacillus* sp. cell surface (12) and opens up the possibility for its use as an antigen carrying vector (13). S-layer proteins are very efficiently expressed and the expression and secretion signals of *slp*A have been used in heterologous protein production systems (14).

Signal peptide that carries protein outside the membrane is required for extracellular expression and secretion. pPG612.1, a secretion vector having *xyl*A promoter with *slp*A signal sequence was used to secrete VP2 polypeptide by using *L. casei* ATCC 393 for porovirus infection. For *In vitro* growth of pSLP111.3 in *Lactobacillus*, xylose is required in the MRS broth and for *in vivo* it was not required (67).

Therefore constitutive promoter with heterologous signal sequence is need of the hour. Both these properties are required for construction of vector to secrete the gene extracellularly.

FedF-PrtP fusions, directed by the signal sequence of *L. brevis Slp*A, were throughout found to be secreted at significantly higher quantities than corresponding fusions with the signal peptide of *L. lactis* Usp45, using Nisin promoter (68). *L. brevis* derived *Slp*A was used as a signal peptide to secrete beta lactamase (*bla)* gene of *E. coli* in different strains of lactic acid bacteria i.e. *L. lactis, L. brevis, L. plantarum, L. gasseri* and *L. casei.* The highest yield of *bla* was obtained with *L. lactis* and *L. brevis*. *L. lactis* produced up to 80 mg/l of *bla* which represents the highest amount of a heterologous protein secreted by LAB so far. Such a high rate was also observed with *L. plantarum,* whereas in *L. brevis* the competition between the wild type *slp*A gene and the secretion construct probably lowered the rate of *bla* production.

The result obtained indicates wide application of the *L. brevis slp*A signals for efficient protein production and secretion in LAB (69). IFN- 2 α and 2 β was expressed in *L. lactis* using heterologous signal peptide *slp*A and *nis*A as heterologous promoter (70).

Various studies have been reported using *Ldh*, a very strong constitutive promoter (7,8). The activity of this promoter is only marginally affected by energy source used to grow the bacteria (7). *Ldh* from *L. agilis* 3 is strongest constitutive promoter among other constitutive promoters like p*slpA,* p23, p144, *pcys*k, *ppg*m and *phl*b (9). The ldh was successfully used to express the gfp gene in *L. reuteri* strains (10).

Gory et al., (2001) expressed *gfp* in *L. sakei* RV1040 by constructing a shuttle vector pRV85, pRV86(2). In this plasmid increased *gfp* expression was due to presence of strong constitutive promoter *ldh*, derived from *L. sakei* 23K. pRV85 vector is also used for *gfp* expression in *L. plantarum* BCC9546 (71) and *L. rhamnosus* GG (72). Hence it can be concluded that increased gfp expression is mainly attributed to the presence of a strong constitutive promoter *Ldh* and the vector pRV85 is a wide host range shuttle cloning vector. Whereas pRV86 has lacL and lacM region homologous to *L. sakei* genomic DNA, which helps the vector to integret into *L. sakei* chromosome.

3.HYPOTHESIS

Lactobacilli are widely used in probiotic preparations and are the non-pathogenic members of the gastrointestinal tract. Recently, several recombinant Lactic acid bacteria (LAB) secreting heterologous proteins have been developed for biotherapeutic application.

The study is designed to develop a secretion vector having a dual function of strong constitutive promoter as well as heterologous signal sequence. The vector would be having a heterologous signal sequence, *slpA* from *Lactobacillus brevis*, with high secretion efficiency and a very strong constitutive promoter *Ldh*. This would thereby help in increased extracellular secretion of protein of interest.

Probiotic lactobacilli are used in treatment of UTI but are inefficient in complete inhibition of the pathogen. The anti-microbial activity of these probiotic lactobacilli can be increased by incorporating anti-microbial peptides such as Colicin E2.

For expression study the constructed vectors will be electroporated in probiotic strain of *Lactobacillus*, isolated from rat fecal sample. The colonization of probiotic strains could be easily monitored with green fluorescent protein (*gfp*), which would be achieved by incorporation of pRV85 plasmid in *Lactobacillus* isolated from rat fecal sample.

The probiotics isolated from tropical fresh water species are efficient probiotics for disease prevention measure in aquatic farms than isolated from other sources. In this study, probiotic characterization of lactic acid bacteria isolated from the small intestine of tropical freshwater fish was explored.

4.OBJECTIVES

a) Isolation of probiotic strains of Lactic acid bacteria from intestine of fish Rohu (*Labeo rohita*).

b) Construction of shuttle secretion vectors, and incorporation of ColE2 gene.

c) (i)Electroporation of pRV85 into fecal isolate SRN3

(ii)Electroporation and determination of constructed vectors pRV86-ColE 2 and pRV86-*slp*A into fecal isolates SRN3 and SRN4.

5.MATERIALS AND METHODS

Isolation of antimicrobial protein producing probiotics strains of LAB

5.1. Sample Collection

Fresh water fish Rohu (*Labeo rohita*) was procured from Himatnagar (Gujarat) fresh water fish hatchery. The fishes were killed, and the surfaces were washed with 0.1% benzalkonium chloride for 1 min to remove external bacteria. Under sterile conditions, the gut region was dissected and homogenized with an appropriate volume of sterile 1% peptone water. Serial dilutions were made up to 10^{-6} dilution, from which 100 µl aliquots were spread MRS agar plates.

5.1.1. Bacterial Strains, Growth Conditions, and Media

All the LAB isolates and standard strain of *L. acidophilus* NCDC15 were grown in MRS medium at 37˚C for 24-42 hours without shaking condition. All the pathogens were grown in Nutrient broth medium at 37˚C for 24 hours in shaking condition. The bacterial strains used in this study are described in Table 2.

5.1.2. Isolation of LAB

Rat fecal sample and sample from fish intestine were collected using sterile forcep and isolation process was followed as described by (73*)*. The samples were suspended in Normal saline for 2 hours. Supernatant was enriched in MRS broth(pH 2.5) kept at 37˚C for 2 hours and plated on MRS+BCP (Bromocresol purple, 0.17g/L) and MRS+Vancomycin

(20µg/mL) agar plate, incubated at 37°Cfor 24-72 hours. Confirmative test of lactobacilli was performed according to Bergey's manual of determinative bacteriology, 9th ed. 1956. Then catalase test and Gram's staining of all the isolates were performed.

Table 2: The bacterial strains used in this study are described in following table:

Strains	Description	Source
Lactobacillus acidophilus	Standard reference strain for probiotics studies	NCDC15
SRN3	Lab isolated strain of *Lactobacillus*	Rat fecal sample
SRN4	Lab isolated strain of *Lactobacillus*	Rat fecal sample
SSR11	Lab isolated strain of lactic acid bacteria	Fish small intestine
SSR14	Lab isolated strain of lactic acid bacteria	Fish small intestine
SSR16	Lab isolated strain of lactic acid bacteria	Fish small intestine
B. subtilis	Pathogen used in antimicrobial and coaggregation assay	MTCC729
S. flexnerii	Pathogen used in antimicrobial and coaggregation assay	MTCC1457
S. Typhimurium	Pathogen used in antimicrobial and coaggregation assay	MTCC733
S. paratyphi	Pathogen used in antimicrobial and coaggregation assay	MTCC735
UPEC	Pathogen used in antimicrobial and coaggregation assay	MTCC443
A. hydrophilla	Pathogen used in antimicrobial and coaggregation assay	MTCC1739
P. aeruginosa	Pathogen used in antimicrobial and coaggregation assay	MTCC1688
E. coli	Pathogen used in antimicrobial and coaggregation assay	MTCC729

5.1.3. Acid and bile tolerance test

Acid and bile tolerance test was performed as described by (73). All the isolates were inoculated in 10 mL MRS broth, pH adjusted to 2.5 with concentrated HCL as well as in

MRS broth with 0.3% bile acid (Oxgall) accordingly. After 2 hours of incubation at 37°C cultures were spread on MRS plates (73).

5.1.4. Antibiotic susceptibility test

To determine susceptibility of the isolates against various antibiotics this test was done. The absorbance of all the isolates was taken at 600nm and 0.132 O.D was set. The isolates were inoculated into 1% MRS broth, which was overlaid on 3% Nutrient base agar. After the solidification of top agar Antibiotic strip (Himedia-OD043R) was placed over the top agar, and plates were incubated at 37˚C for 24-48 hours.

5.1.5. PCR based identification of the isolates

Strain identification at molecular level was done by performing PCR with *Lactobacillus* universal primers (LactoF and LactoR) (74). Strain identification of probiotic LAB isolates from fish intestine was done by performing PCR with primers for virulence gene

.(75).Strain determination of coccoid LAB is yet to be determined at molecular level by performing PCR with specific 16SrRNA primers.

Table 3: Primers for PCR reaction of isolates:

Primer	**Primer Sequence (5'-3')**	**Tm (°C)**	**Product Size (bp)**
Lactobacillus universal primer			
LactoF	TGGAAACAGRTGCTAATACCG	57	232
LactoR	GTCCATTGTGGAAGATTCCC		
Enterococcus virulence gene gelE primer			
Forward	ACCCCGTATCATTGGTTT	57	232
Reverse	ACGCATTGCTTTTCCATC		

Table 4: Components of PCR reaction:

Components	Volume (µl)
PCR master mix	12.5
Forward primer	1
Reverse primer	1
Genomic DNA (SRN3, SRN4, SSR11 ,SSR14, SSR16)	5
Nuclease free water	5.5

5.1.6. Probiotic characterisation of LAB isolates

5.1.6.1. Bacterial strains and growth condition

SRN3 and SRN4, lab isolated strains of lactobacilli and SSR11, SSR14 and SSR16 lab isolated, Lactic acid bacteria strains were studied for their probiotic activity. The cultures were grown in MRS (de Man Rogosa Sharpe) media at 37˚C for 48-72 hours. Pathogens used in coaggregation study are described in Table 2. Bacterial cultures (Isolates and pathogens) were harvested by centrifugation at 6000 rpm for 10 min, washed twice in sterile PBS (pH 7.0) and resuspended in PBS to an OD_{600} of 0.6. The final suspension was used for probiotic assays.

5.1.6.2. Cell surface hydrophobicity Assay

The surface hydrophobicity of the lactobacilli was determined by measuring the affinity of cells cultured overnight for xylene or toluene in a two-phase system as described by Eckmecki et al (2009).

The percentage cell surface hydrophobicity was calculated using the following equation:
$H\% = [(OD_{600}$ before mixing - OD_{600} after mixing)/(OD_{600} before mixing)] * 100

5.1.6.3. Autoaggregation Assay

The isolated bacterial suspensions made in PBS were incubated at 37˚C and O.D at 600nm was measured at 1, 2, 4 and 6 hours of time intervals. Autoaggregation was calculated from three replicates as the percent decrease in optical density of the original bacterial suspension due to cell clumping.

% Autoaggregation of the two strains were deterimined by following formula: $A\% = [(A_0 - A_t)/A_0]*100$

A_0 =Absorbance at 600nm at t_o time

A_t = Absorbance at 600nm at different time intervals (1, 2, 4 and 6 hours).

5.1.6.4. Coaggregation assay

Coaggregation assay was done as described by Eckmecki et al., 2009. Optical density of the original bacterial suspension due to clump formation between isolates and pathogens will be decreased.

% Coaggregation between lactobacilli and pathogens were determined by following formula:

$C\% = 100 \times [(OD_1 + OD_2) - 2(OD_3)]/(OD_1 + OD_2)$

OD_1 = Absorbance at 600nm at t_o time of *Lactobacillus* strain

OD_2 = Absorbance at 600nm at t_o time of pathogen strain

OD_3 = Absorbance at 600nm at t (1, 2, 4, 6 hours) time interval of mix culture (*Lactobacillus* strain+pathogenic strain)

5.1.7. Determination of antimicrobial activity of LAB isolates

The assay was performed as described by (76). Active culture of pathogen was prepared by inoculating the pathogen in L.B broth, incubated at 37˚C for 24 hours in shaking condition. On the next day 1 mL of active culture of pathogen was inoculated into 9 mL of L.B broth. The pathogens were allowed to grow in L.B broth for 3 hours and after each hour O.D at 600nm was noted. On beginning of 3rd hour 1 mL cell free supernatant (CFS) of both the isolates were inoculated into the active culture of pathogen respectively. The O.D was determined at 600nm after each hour of 4 hour incubation time period. The similar kind of assay was performed for LAB isolates from fish. The pathogenic strains mentioned in Table 2, were set at 0.132 in $O.D_{600}$, which was subsequently mixed in 1% top agar, and poured over 3% base agar (N. agar). Upon solidification, 2mm diameter wells were punctured in which 200µl of cfs of the isolates was added. The plate was incubated at 37˚C overnight to examine the presence of inhibition zone surrounding the well.

5.1.8. Isolation and purification of crude antimicrobial protein from CFS of isolates

The isolated strains were grown in 300 mL MRS broth (pH 6.0) seeded with 5 % inoculum of overnight culture and were incubated at 30˚C for 48 h. Cells were then removed from the growth medium by centrifugation at 10,000 rpm for 20 min at 4˚C. Purification of antimicrobial protein was achieved by using a multistep protocol.

5.1.8.1. Ammonium Sulfate Precipitation

Culture supernatant was brought to 90 % saturation with solid ammonium sulfate, and after stirring overnight at 4˚C, the precipitate was collected by centrifugation (10,000 rpm, 10 min, 4˚C). The obtained black coloured pellets were dissolved in 50mM sodium phosphate buffer and mixed with 60 mL (2:1) chloroform: methanol solution incubated at 4˚C for 1 hour. White precipitates were collected by centrifugation at 10,000 rpm at 4˚C for 25 min.

The pellet was air dried and dissolved in sodium phosphate buffer. The obtained solution was of partially purified proteins present in cfs. The presence of bacteriocin in crude protein of SRN4 was determined by checking its antimicrobial activity against *A. hydrophila.*

5.1.8.2. Protein estimation

Protein estimation was done by Folin Lowry method (77). 5 mL of alkaline solution (50 mL of 2% Na_2CO_3 in 0.1M NaOH and 1mL of 0.5% $CuSO_4.5H_2O$ in 1% Na-K tartrate) was mixed with 1 mL of test solution and 1 mL of standard protein, and were mixed thoroughly and were kept for 10 minutes at room temperature. Then 0.5 mL of 1N Folin ciocaltue reagent was rapidly added with immediate mixing. Then it was incubated in dark for 30 minutes and OD at 750 nm was taken.

5.1.8.3. SDS-PAGE

The molecular weight of partially purified bacteriocins of all the five isolates were analyzed by SDS-PAGE as directed by (78) using 10-20% gradient gel.

5.1.8.4. Reverse-Phase High-Performance Liquid Chromatography of SRN3 and SRN4 crude protein

The crude protein sample of SRN4 was resuspended in water containing 0.1% trifluoroacetic acid (TFA). This was resolved on analytical C-18 reverse phase column (100×2.5 mm; ACE Capillary Column,Advance Chromatography Technology, Scotland) using an HPLC system (Agilent,USA). The column was equilibrated with solvent A (HPLC-grade water containing 0.1% TFA) and fractions were eluted with a step gradient 0-100% of solvent B (100 % acetonitrile containing 0.1% TFA) for 50 min. Flow rate was maintained at 0.25 mL/min and temperature (60 °C) was maintained and eluted analytes were monitored by an ultraviolet detector at 210 nm.

5.1.8.5. Size Exclusion Chromatography

The SRN 4 crude protein was purified by size exclusion chromatography. SRN4 crude protein was loaded on a Superdex 75 (10/300 GL) prepacked Tricon column (GE Healthcare, USA) linked to a high-performance liquid chromatography (HPLC; Agilent, 1100) system equilibrated with sodium phosphate buffer (pH 6.7) with 200 mM NaCl (78) and eluted peak was collected in 24 fraction, nomenclatured as F1 to F24.

Construction of shuttle secretion vectors, incorporation of ColE2 gene and electroporation of GFP containing vector in SRN3 and SRN4.

5.2. Bacterial strains and growth conditions used for genetic modification studies

The strains used in this study are shown in Table 2. *E. coli* DH5α and *E. coli* BL 21 cells used for cloning were grown in LB medium (Himedia, Mumbai) at 37˚C with shaking. Antibiotics such as ampicillin, erythromycin and chloramphenicol (Himedia, Mumbai) were used for *E. coli*. The genetic modification will be performed in Lab isolated bacteriocin producing probiotic strains SRN3 and SRN4.

Table 5: Bacterial strains used in genetic modification study

Strains	Description	Source
E. coli DHα	Transformation host	MTCC, Chandigarh
*E. coli*BL21 *(D3)*	Transformation host for protein expression	MTCC, Chandigarh

SRN3	Test host	Lab isolate
SRN4	Test host	Lab isolate

5.2.1. Plasmids:

Table 6: Plasmid shuttle vectors for *Lactobacillus* and *E. coli* used in this study.

Vector	Description	Referance
pColE2-P9	Stp^r, vector for colicin E2	The Coli Genetic Stock Center, Yale University
pSLP111.3	Cm^r, Secretory vector having slpA signal peptide	Gift from Jos Seegers, Lactrys, Netherlands
pRV85	Em^r, Expression vector having LDH promoter and *gfp*	Gift from Prof. M. Champomier Verges, INRA, France
pRV86	Em^r, Amp^r, Expression vector having LDH promoter and *gfp*	Gift from Prof. M. Champomier Verges, INRA, France

5.2.1.1. Plasmid pRV85

The plasmid pRV85 is a type of shuttle expression vector of 6.2kb size, containing a strong constitutive promoter LDH, Gfp as a marker gene. It also provides resistance against Erythromycin. The vector was kindly gifted by Prof. M. Champomier Verges, INRA, France

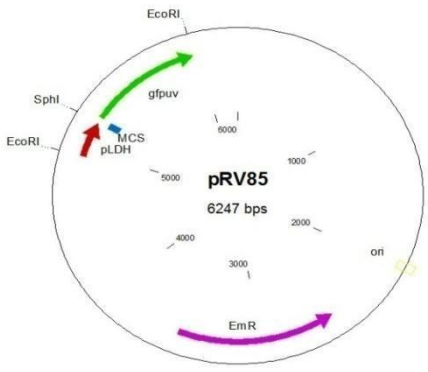


Figure 4: pRV85 vector

5.2.1.2. Plasmid pRV86

The plasmid pRV86 is a type of chromosomal integrated shuttle expression vector of L. sakei of size 6.3kb, containing a strong constitutive promoter LDH, Gfp as a marker gene. It also provides resistance against erythromycin and ampicillin. The vector was kindly gifted by Prof. M. Champomier Verges, INRA, France

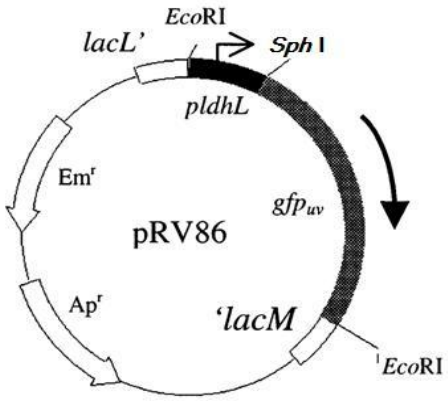


Figure 5: pRV86 vector

5.2.1.3. Plasmid pColE2-P9

Plasmid pColE2-P9 is an *E.coli* plasmid of size 7.2kb encoding Colicin E2 gene and streptomycin resistant marker. The vector was kindly gifted by the Coli Genetic Stock Center, Yale University.

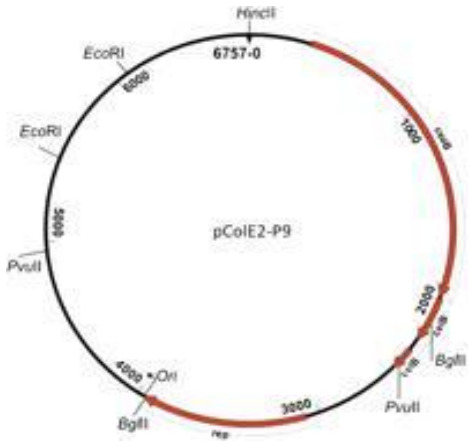


Figure 6: pColE2-P9 vector

5.2.1.4. Secretion Vector pSLP111.3

The plasmid pSLP111.3 is a type of secretion expression vector of 4.1kb size, containing xylA promoter, *slp*A as secretion signal and cell wall anchor domain, were kindly gifted by Jos Seegers (Falco Biotherapeutics, Netherlands).

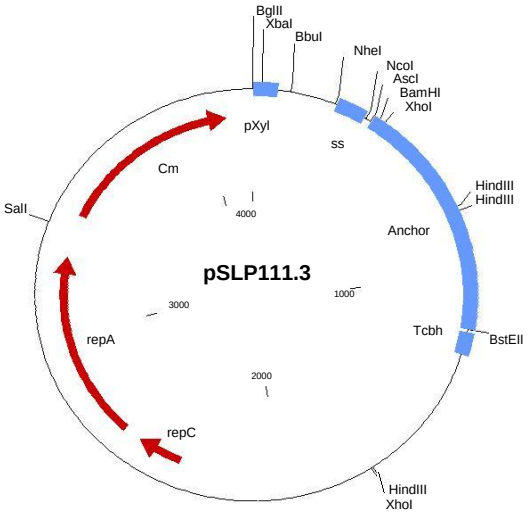


Figure 7: pSLP111.3 vector

5.2.2. Construction and cloning strategy:

5.2.2.1. Strategy 1: Incorporation of colicin gene (ColE2) in pRV86

Colicin gene fragment of about 2 Kb was obtained from the plasmid of pColE2-P9 by polymerase chain reaction (PCR) amplification with the primers 5'-GGATCCATGAGCGGTGGCGAT -3' (forward) containing a *Bam*HI site (underlined) and 5'-CTCGAGTCAGCCCTTTTAAATCCTGA-3'(reverse) containing an *Xho*I site (underlined). The PCR product of Colicin E2 gene was inserted into the blunted and alkaline phosphatase treated *Sph*I digested pRV86 and blunt end ligation was performed.

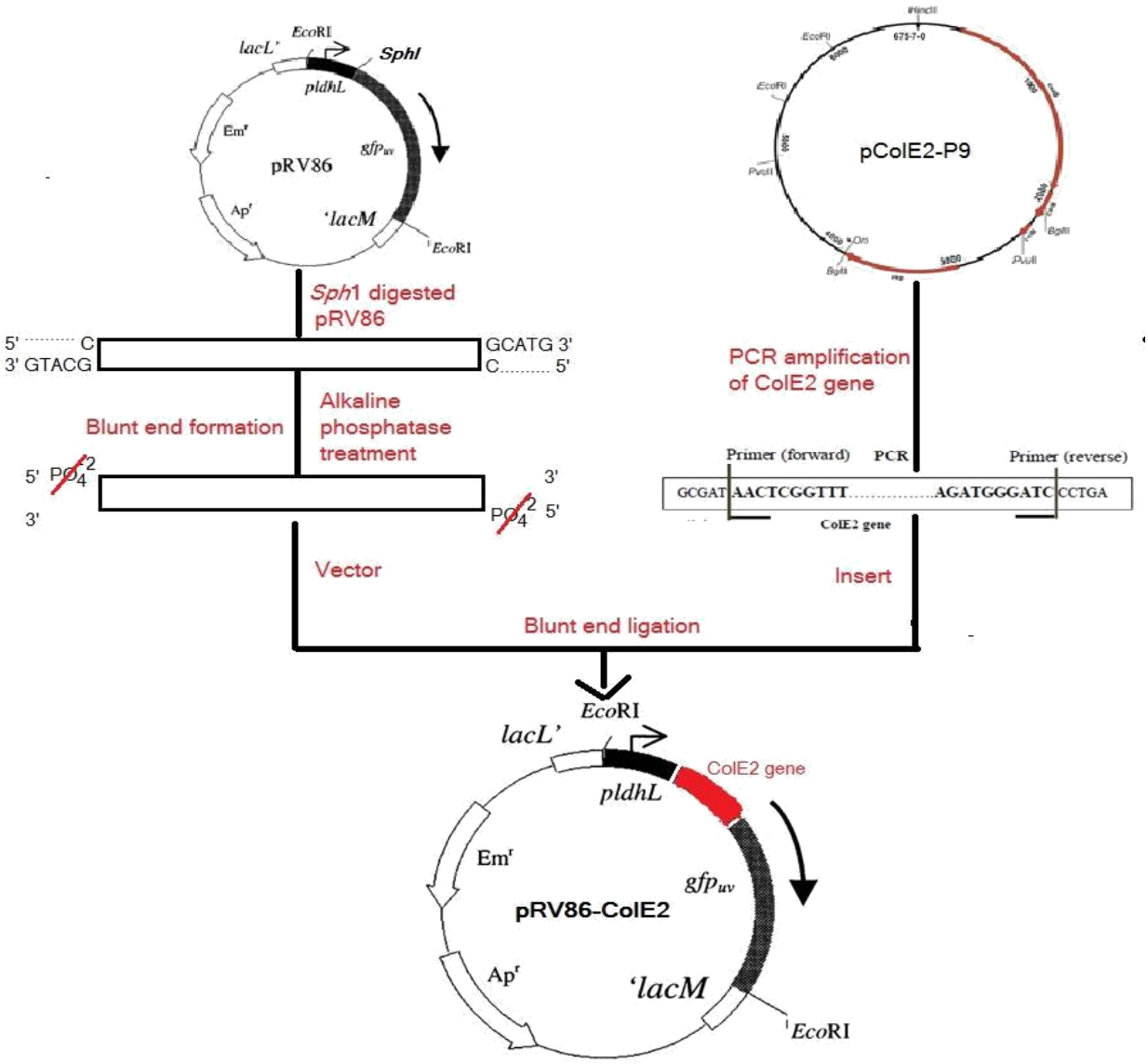


Figure 8: The construct of recombinant vector expressing Colicin E2.

The ColE2 gene (*ceaB*) and its immunity gene (*ceiB*) on plasmid DNA of (pColE2-P9) were amplified using the polymerase chain reaction (PCR) with the primers. Vector pRV86 was digested with *Sph*I restriction enzyme and blunt ends were formed by using T4 DNA polymerase 3' to 5' exonuclease activity by Quick blunting Kit (New England Biolabs, UK). To avoid self ligation alkaline phosphatase enzyme treatment was also given to blunted vector. The blunt end ligation of the ColE2 insert and pRV86 was performed by using Rapid DNA ligation Kit (Fermentas).

5.2.2.2. Strategy 2: Incorporation of *slp*A signal peptide in pRV86

Secretion vector pSLP111.3 was RE digested with *Nhe*I and *Hind*III. Three fragments of different sizes were obtained. Among them 503 bp fragment was having *slp*A signal peptide and having 5' overhangs .The signal peptide was blunted by 5' to 3' polymerase activity of T4 DNA polymerase and blunt end ligation was performed with already blunted and alkaline phosphatase enzyme treated pRV86 vector.

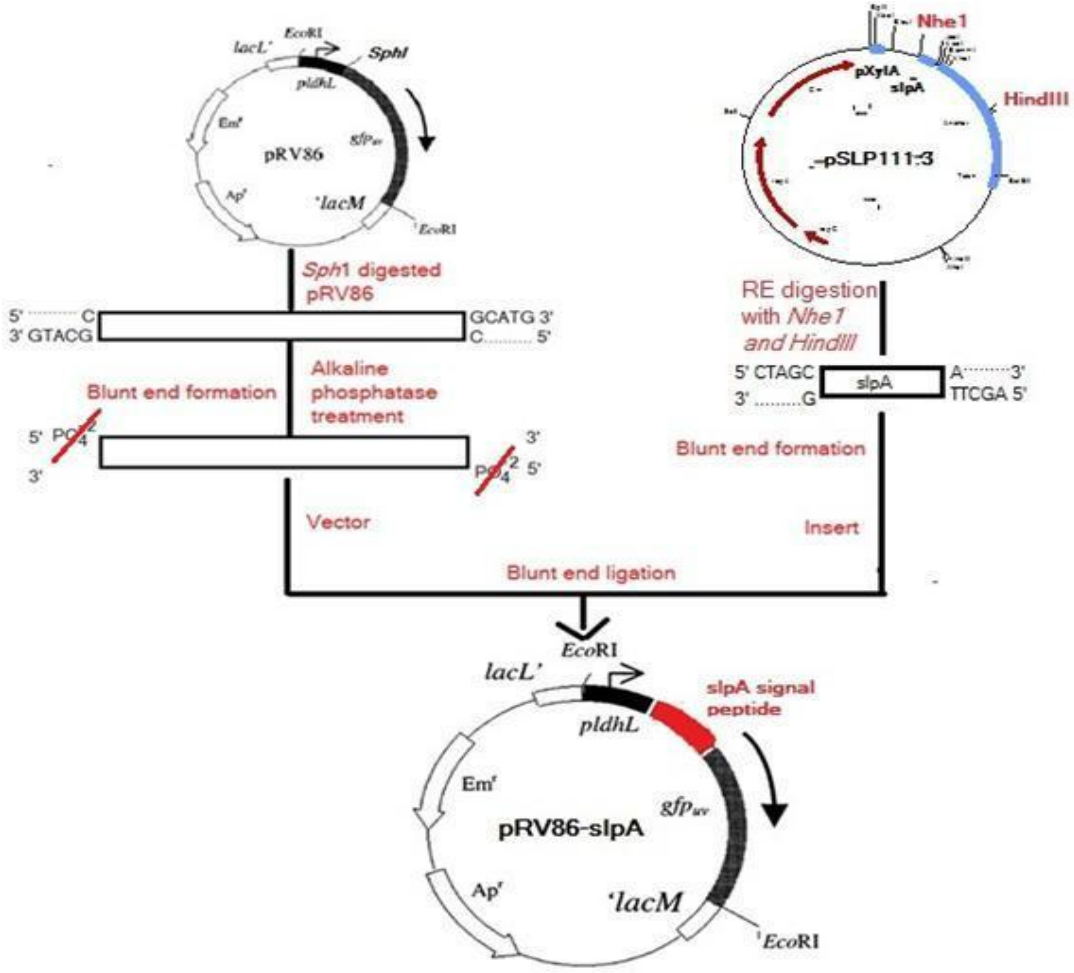


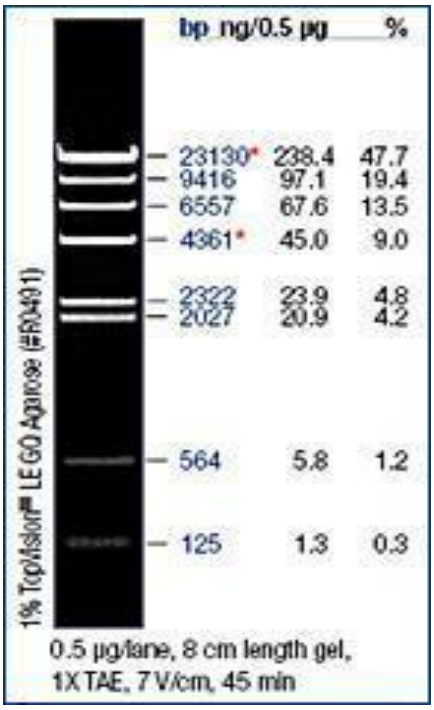
Figure 9: The construct of recombinant vectors expressing *slp*A signal peptide.

5.2.2.3. Strategy 3: Transformation of GFP containing shuttle expression vector pRV85 in lab isolated probiotic strains of lactobacilli i.e. SRN3 and SRN4 via electroporation.

The electroporation of pRV85 in competent lactobacilli isolates was followed according to the protocol of (79).

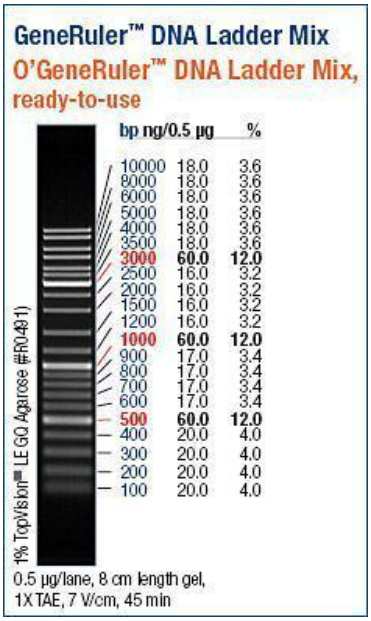
5.2.3. DNA Marker

λ DNA *Hind*III digest (Fermentas)



Lambda DNA/*Hind*III Marker, 2, is premixed with DNA Loading Dye at a final DNA concentration of 0.1µg/µl and can be directly applied onto an agarose gel. The DNA marker contains the following 8 discrete fragments (in base pairs): 23130, 9416, 6557, 4361, 2322, 2027, 564, 125.

Gene Ruler DNA Ladder Mix



The DNA ladder contains 21 discrete fragments from 100 bp to 10000 bp.

5.2.4. Transformation of Plasmid DNA into *E.coli* using calcium chloride method

Transformation of plasmid DNA into *E.coli* was done by calcium chloride method as per the protocol (80).

5.2.5. Plasmid isolation by Alkaline Lysis Method (MINIPREP)

Plasmid isolation of pRV85, pRV86, pColE2-P9 and pSLP111.3 was performed by using the protocol (80).

5.2.6. DNA fragment was excised from an agarose gel using Sure Extraction/PCR Clean Up Kit.

DNA fragment was excised from an agarose gel using Sure Extraction/PCR Clean Up Kit.

5.2.7. Restriction digestion

All the enzymes used in the study are from New England Biolabs and Thermo scientific.

Table 7: Restriction enzymes used in this study

Restriction enzymes	Concentration (U/µl)	Source
*Nhe*I (HF)	10	NEB
*Xho*I	10	NEB
*Hind*III	10	NEB
*Sph*I	10	Thermo scientific
*Eco*RI	10	Thermo scientific
*Bam*HI	10	Thermo scientific

5.2.7.1. Restriction digestion of pRV86:

Table 8: Components of Restriction digestion system for pRV86

Components	Volume (µl)	
	Control	Test
Nuclease free water	15	14
10x buffer B	2	2
DNA (1µg)	3	3
*Sph*I	-	1
Total volume	20	20

The reaction mixture was incubated at 37˚C for 1 hour and inactivated at 65˚C for 20min.

5.2.7.1.1. Blunt end formation of pRV86:

The 3’ overhangs of *Sph*I digested pRV86 was blunted by Quick blunting kit (New England biolabs, UK). The DNA is blunted by using 3’ to 5’ exonuclease activity of T4 DNA polymerase.

Table 9: Components of blunt end formation for pRV86

Components	**Volume (µl)**
Purified DNA	7
10X blunting buffer	2
1Mm dNTP mix	2
Blunt Enzyme mix	1
Nuclease free wate	8
Total volume	20

The reaction mixture was incubated at 25˚C for 15 min and immediately inactivated by heating at 70˚C for 10 min.

5.2.7.1.2. Alkaline phosphatase treatment of blunt ended pRV86

The 5’ phosphate group was removed by Thermo Scientific FastAP Thermosensitive Alkaline Phosphatase to avoid self ligation of pRV86.

Table 10: Components of alkaline phosphatase treatment for pRV86

Components	**Volume (µl)**
Blunted vector	20
10X buffer for AP used in reaction	2.5
FastAP Thermosensitive Alkaline Phosphatase	1
Nuclease free water	1.5
Total volume	25

The reaction mixtures was mixed, spinned down briefly and incubated 10 min at 37˚C. The

reaction was stopped by heating for 5 min at 75˚C.

5.2.7.2. Restriction digestion of pSLP113.3

Table 11: Components of Restriction digestion system for pSLP111.3

Component	**Volume(µl)**	
	Control	**Test**
BSA	_	0.5
DNA	3	3
*Nhe*I	_	1
*Hind*III	_	1
Sterile distilled water	42	39.5
10X NEB buffer	5	5
Total volume	50	50

The reaction mixture was incubated at 37ºC for 1 hour. After 1 hour, the restriction enzymes were inactivated by heating at 65 ºC for 20 min.

Blunt end formation of *slp*A

The following components were mixed in a sterile microfuge tube.

Table 12: Components of Blunt end formation of *slp*A

Components	**Volume (µl)**
Purified DNA	30
10Xblunting buffer	4
1mM DNTP mix	4
Blunting enzyme mix	1
Sterile water	1
Total volume	40

The reaction mixture was incubated at 25˚C for 15 min and immediately inactivated by

heating at 70˚C for 10 min.

5.2.8. Polymerase Chain Reaction

PCR was used to amplify the ColE2 gene from pColE2-P9.

A PCR reaction involves the following components:

Table 13: Components of PCR reaction

Components	**Volume (µl)**
Master mix	12.5
Forward primer	1
Reverse primer	1
Plamid DNA	6
Nuclease free water	4.5

PCR reaction for ColE2

The PCR condition used for amplification of Col E2 was 30 cycles of 30 s at 94ºC, 30 s at 58 ºC, 2 min 20 s at 72 ºC after denaturing for 4 min at 94 ºC.

5.2.9. Ligation

Ligation of Colicin E2 and slpA was done using the Rapid DNA Ligation Kit which enables sticky end or blunt end DNA ligation by T4 DNA ligase as followed:

Table 14: Components of ligation reaction of Colicin E2 gene

Components	**Volume (µl)**
Purified DNA	30
10Xblunting buffer	4
1mM DNTP mix	4
Blunting enzyme mix	1
Sterile water	1
Total volume	40

The reaction mixture was incubated at 25˚C for 15 min and immediately inactivated by

heating at 70˚C for 10 min.

Ligation of *slp*A was done using the Rapid DNA Ligation Kit as followed:

Table 15: Components of ligation reaction of *slp*A gene

Components	**Volume (µl)**
Linear pRV86 vector	2
Insert DNA (*slp*A)	20
5X ligation buffer	6
T4 DNA ligase	1
Sterile water	1
Total volume	30

The reaction mixture was incubated at 22ºC for 5 min and 5 µl of ligation mixture was used

for transformation.

5.2.10. Electroporation

The electroporation of pRV85 in competent lactobacilli isolates was followed according to the protocol of (79).

6.RESULTS

Isolation of probiotic strains of Lactic acid bacteria from rat fecal sample and fish intestine.

6.1. Isolation and identification of LAB from rat fecal sample

The total number of colonies obtained from MRS+BCP (0.17g/L) plates were 36, among them 14 colonies based on its morphology were further being streked on MRS+Vancomycin plates and 8 colonies showed resistance against vancomycin. Out of the 8 colonies, 5 colonies could grow on 2.5 pH adjusted MRS agar plate as well as MRS+0.3% bile (Oxgall) agar plate. All the 5 selected isolates, labeled as SRN1-5, were catalase negative and Gram positive rods (Fig.10).

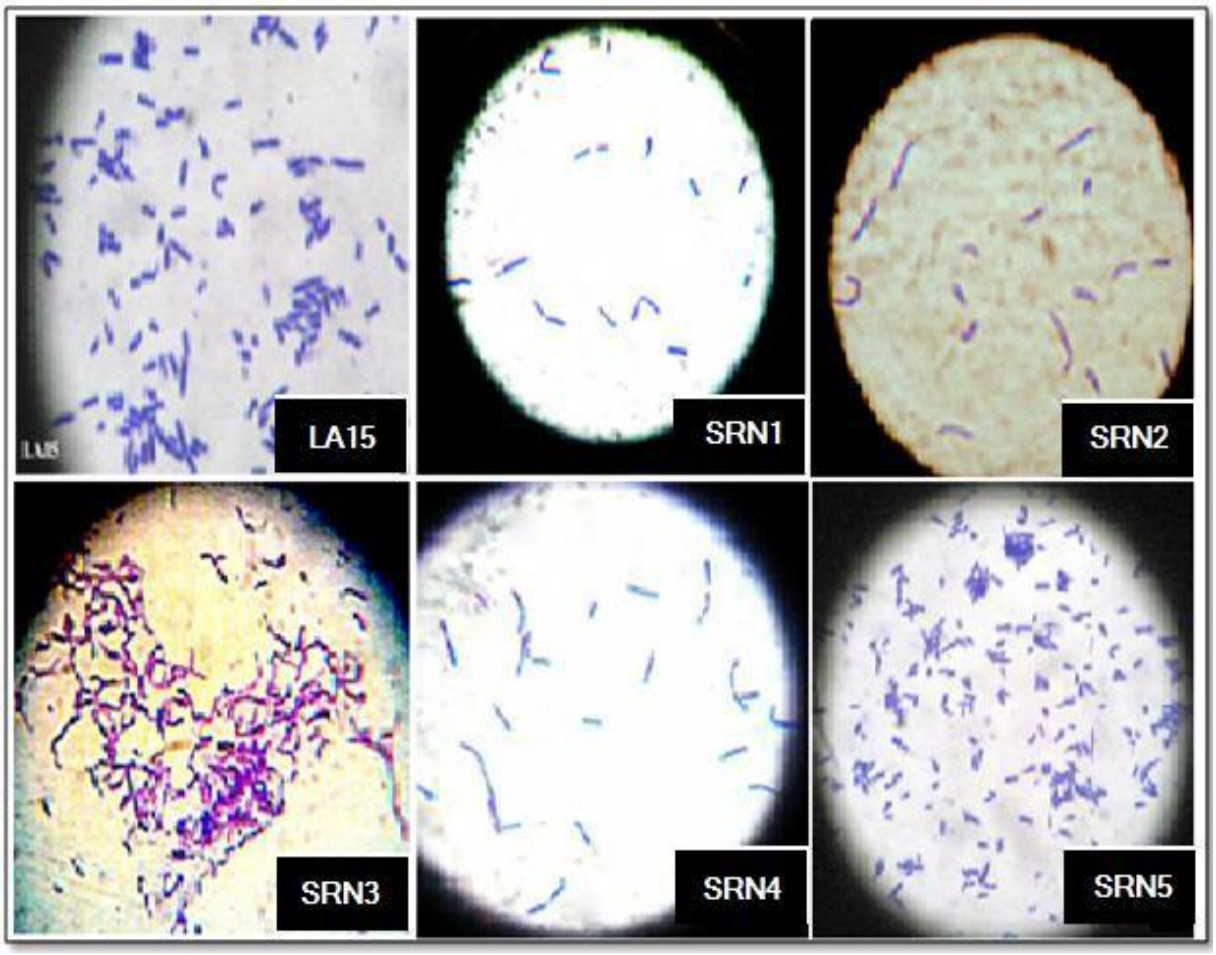


Figure 10: Gram staining of *L. acidophilus*NCDC15 standard strain and LAB isolated from rat fecal sample

6.1.1. Antibiotic susceptibility test

Each of the five isolates were tested for their susceptibility against various antibiotics ,

*S*RN3 and SRN4 isolates showed sensitivity against all the antibiotics beside vancomycin and were selected for further probiotic characterization study (Table 16).

Table 16: Antibiogram assay of the isolates SRN1 to SRN5

Isolates	Antibiotics								
	KAN (30mg)	**COT (25mg)**	**AMK (30mg)**	**STR (25mg)**	**VAN (20mg)**	**CRM (30mg)**	**AMP (10mg)**	**TET (30mg)**	**GEN (10mg)**
SRN1	S	S	S	S	R	S	R	S	S
SRN2	R	S	S	S	R	S	S	S	S
SRN3	S	S	S	S	R	S	S	S	S
SRN4	S	S	S	S	R	S	S	S	S
SRN5	S	S	S	S	R	S	S	S	R

S: Sensitive, R: Resistant

6.1.2. PCR based identification of SRN3 and SRN4 with universal *Lactobacillus* primers (LactoF and LactoR)

The genus level confirmation of both the isolates (SRN3 and SRN4) was performed by using universal primers of lactobacilli i.e LactoF and LactoR. A band of 232bp amplicon size was observed of both the isolates i.e SRN3 and SRN4 (Fig.11).

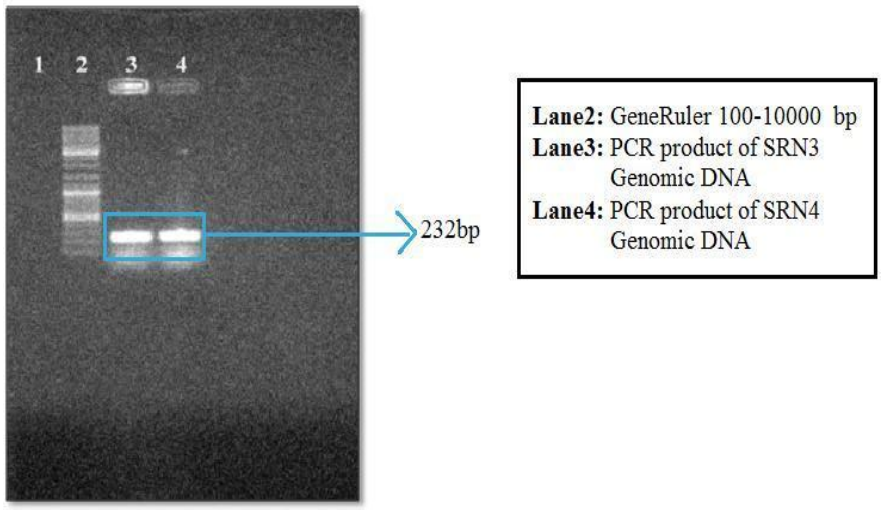


Figure 11: Gel image of PCR amplified region of genomic DNA of lactobacilli isolates.

6.1.3. Probiotic characterization of lactobacilli strains

6.1.3.1. Cell surface hydrophobicity assay

The cell surface hydrophobicity of SRN3 and SRN4 were deterimied by using xylene and toluene as hydrophobic solvents. Both the strains SRN3 and SRN4 showed higher amount of hydrophobicity percentage i.e 48.03±0.89, 51.71±0.92 for xylene and 44.85±0.83, 55.82±0.68 for toluene accordingly as compared to standard strain *L. acidophilus* NCDC15 which showed 37.04±0.43 percent hydrophobicity to xylene and 55.1±0.76 percent hydrophobicity to toluene . Among them SRN4 showed highest amount of % hydrophobicity for both xylene and toluene.

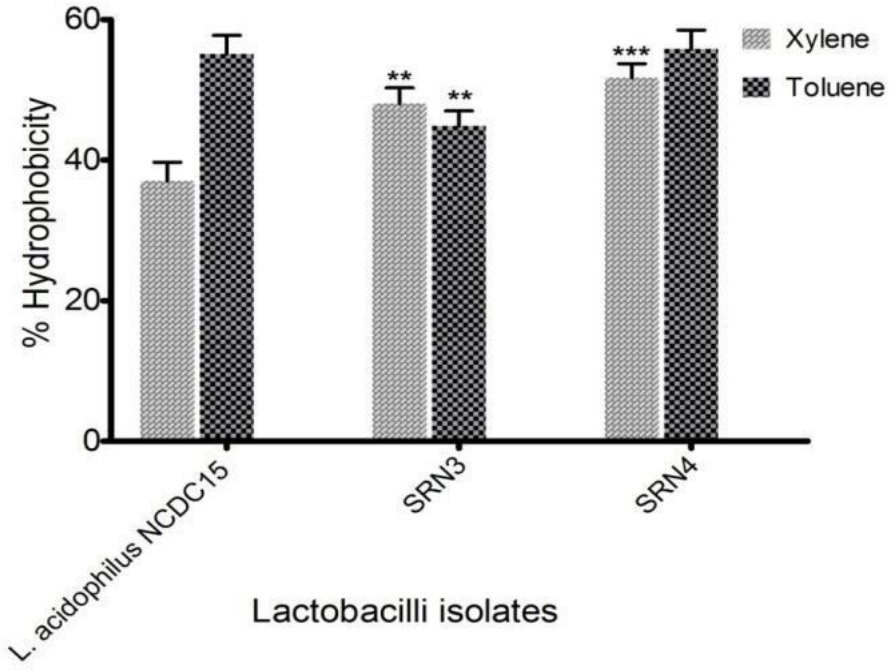


Figure 12: Cell surface hydrophobicity of standard strain and isolated strains. Data are expressed as mean ± SD (n = 3); ** P < 0.01, *** P < 0.001.

6.1.3.2. Autoaggregation assays

SRN3 and SRN4 both the isolated strains were showing higher *autoaggregation* percentage as compare to the standard strain *L. acidophilus* NCDC15 after incubation at 37°C for 6 hours. SRN3 and SRN4 strains were showing 62.43±0.45 and 62.92±0.98 percentage of autoaggregation which is higher than the standard strain *L. acidophilus* NCDC15 (53.96±0.99). This result indicates that the SRN3 and SRN4 possess potential ability to adhere to epithelial cells and mucosal surfaces (Fig.13).

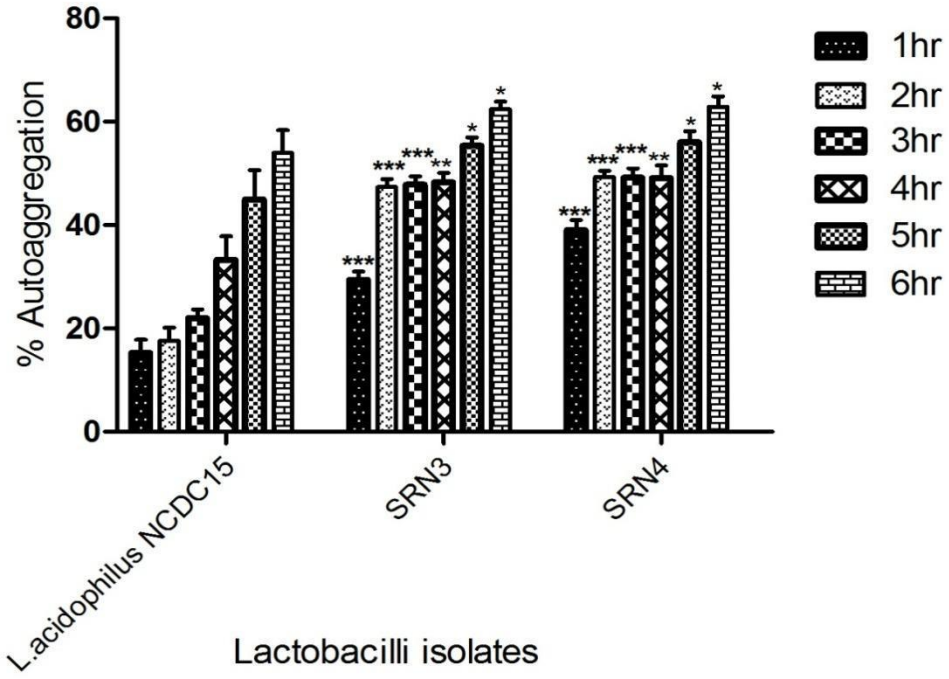


Figure 13: Autoaggregation of standard strain and isolated strains. Data are expressed as mean ± SD (n = 3); * P < 0.05, ** P < 0.01, *** P < 0.001.

6.1.3.3. Coaggregation assay

All tested strains showed some coaggregation properties with pathogens (Fig.14). Among the isolated strains, SRN3 and SRN4 showed better coaggregation with UPEC*P.aeruginosa, A. hydrophila,* and *E. coli* as compared to *L. acidophilus* NCDC15 standard strain.

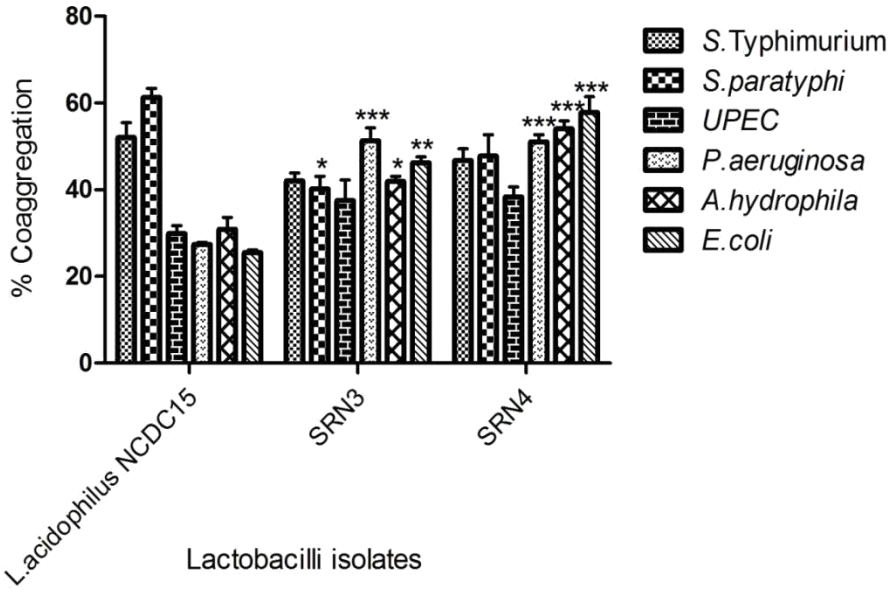


Figure 14: Coaggregation of standard strain and isolated strains with pathogens. Data are expressed as mean ± SD (n = 3); * P < 0.05, ** P < 0.01, *** P < 0.001.

SRN 3 show higher coagregation with *P. aeruginosa* (51.33%), with UPEC (37.48%) with *A. hydrophila*(41.86%) & with *E. coli*(46.26%) than compared with *L. acidophilus* NCDC15 which showed with *UPEC*(29.93%) , with *A. hydrophila*(30.9%) & with *E.coli* (25.50%).

SRN 4 show higher coagregation with *P. aeruginosa* (51.02%), with UPEC (38.36%) with *A. hydrophila*(51.06%) & with *E. coli*(57.84%) than compared with *L. acidophilus* NCDC15 which showed with UPEC(29.93%) , with *A. hydrophila*(30.9%) & with *E. coli* (25.50%).

6.1.4. Determination of antimicrobial activity of SRN3 and SRN4 isolates

Cell free supernatants of SRN3 and SRN4 were incubated with pathogens and their antimicrobial activities were determined. In Fig.15 the normal growth curve of pathogen and the growth of pathogen after adding CFS of the isolates are showed un different coloured lines.

SRN4 strain was showing bacteriocidal activity against *B.subtilis*, and for all other pathogens, the lactobacilli strains SRN3 and SRN4 were showing bacteriostatic activity (Fig.15).

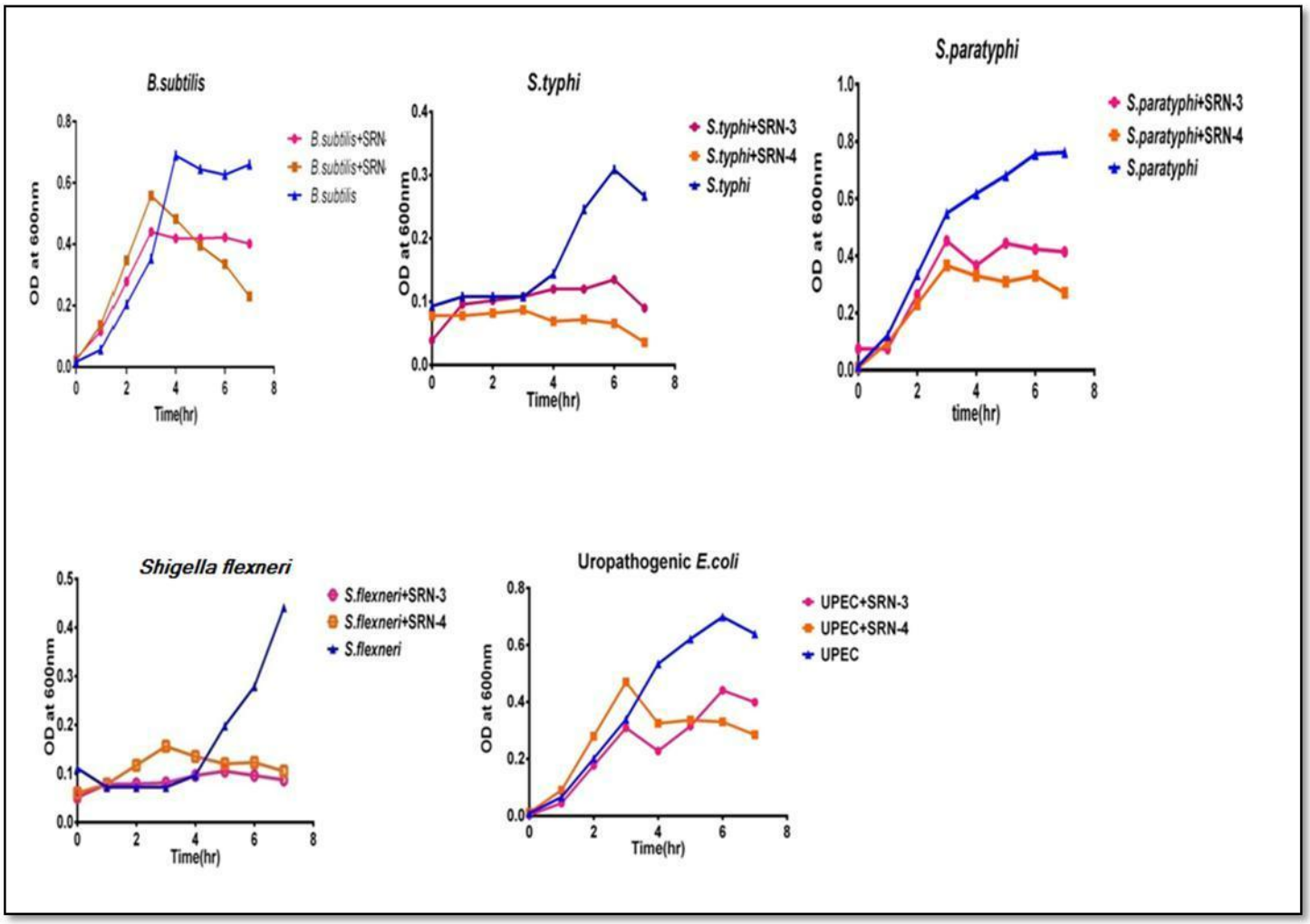


Figure 15: Antimicrobial assay of isolates (SRN3 and SRN4) against pathogens

6.2 Isolation and identification of LAB from fish intestine

A total of 56 colonies were counted and designated as SSR 1– 56. Among them, 30 colonies were selected randomly to test the confirmation of LAB isolates. Out of the 30 colonies 23 colonies could grow on MRS+BCP plates. Among them 8 colonies based on their morphological characters were streaked on MRS+ vancomycin plates. All of the eight

colonies were sensitive to vancomycin. The Gram's staining result confirms the presence of Gram-positive cocci and were selected for further studies (Fig.16).

PCR determination of the isolates with universal *Lactobacillus* primers LactoF and LactoR indicated the absence of lactobacilli strains and with *Enterococcus* toxicity gene *gelE* showed the absence of virulence gene in isolates. But the strain identification of this coccoid LAB isolate through PCR amplification with specific 16SrRNA primers is under process .

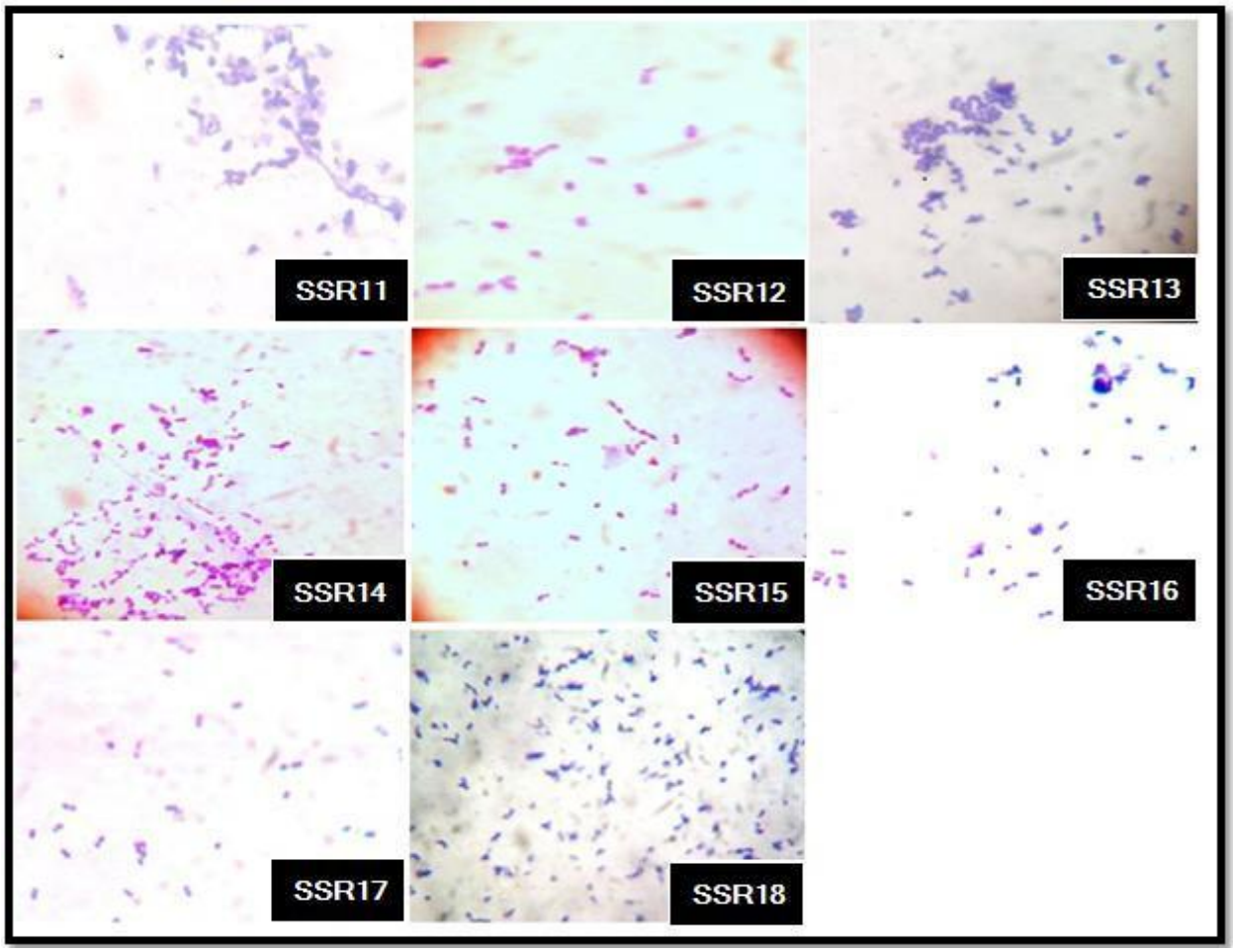


Figure 16: Gram staining of LAB isolated from fish intestine.

6.2.1. Antibiotic susceptibility test

Each of the eight isolates were tested for their susceptibility against various antibiotics, SSR11, SSR13, SSR14, SSR15 and SRN16 isolates showed sensitivity against all the antibiotics. All the five isolates were thus selected for further studies (Table 17)**.**

Table 17 : Antibiogram assay of the isolates SSR11 to SSR18

Isolates	Antibiotics								
	KAN (30mg)	**COT (25mg)**	**AMK (30mg)**	**STR (25mg)**	**VAN (20mg)**	**CRM (30mg)**	**AMP (10mg)**	**TET (30mg)**	**GEN (10mg)**
SSR11	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
SSR12	**R**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**S**
SSR13	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
SSR14	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
SSR15	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**
SSR16	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
SSR17	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**S**
SSR18	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**S**	**R**

S:Sensitive, R: Resistant

6.2.2. Determination of antimicrobial activity of LAB isolates

The antimicrobial assay of isolates SSR11, SSR13, SSR14, SSR15 and SSR16 was done by performing well diffusion assay against various pathogens (Fig.17; Table18).

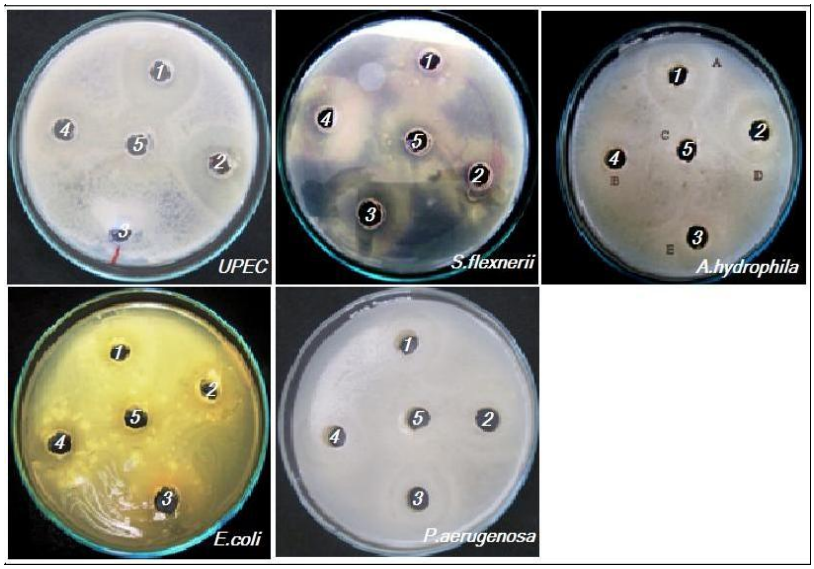


Figure 17 : The antimicrobial assay of isolates **1:** SSR11, **2**: SSR14, **3:** SSR15, **4:** SSR16, **5:** SSR13 against pathogens.

Table 18: Well diffusion assay of isolated strains

Isolates	Zone of inhibition against pathogens (mm)				
	UPEC	*S.flexnerii*	*A.hydrophila*	*E.coli*	*P.aeruginosa*
SSR11	35	33	25	34	–
SSR13	30	–	28	25	–
SSR14	33	33	28	33	–
SSR15	30	–	–	30	–
SSR16	27	30	–	30	26

Based on the antimicrobial activity against the pathogens, 3 out of 5 isolates i.e SSR11, SSR14 and SSR16 were selected for their probiotics characterization.

6.2.3. Probiotic characterization of LAB strains

6.2.3.1. Cell surface hydrophobicity assay

The cell surface hydrophobicity of SSR11, SSR14 and SSR16 were deterimied by using xylene and toluene as hydrophobic solvents. All the three isolates SSR11, SSR14 and SSR16 showed lower amount of hydrophobicity percentage i.e 10.54±0.89, 13.59±0.92, 25.88±0.78 for xylene and 17.26±0.83, 25.33±0.68, 25.41±0.98 for toluene accordingly as compared to standard strain *L. acidophilus* NCDC15 which showed 37.04±0.43 percent hydrophobicity to xylene and 55.1±0.76 percent hydrophobicity to toluene . SSR16 showed. higher amount of % hydrophobicity for both xylene and toluene among all the three isolates.

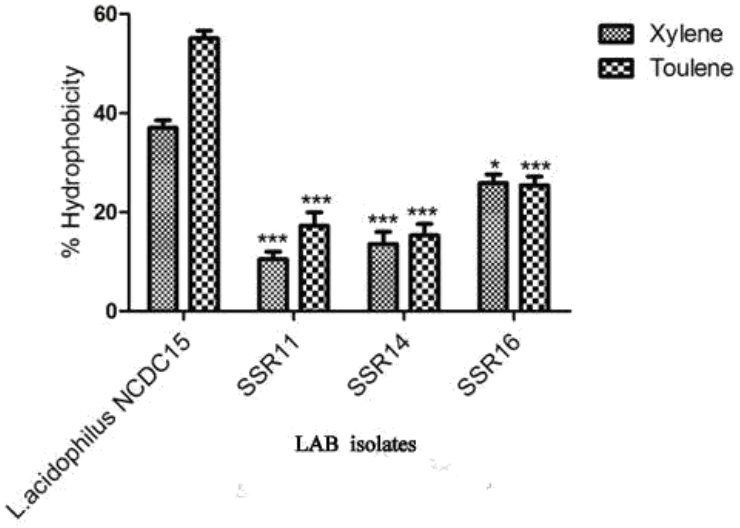


Figure 18 : Cell surface hydrophobicity of standard strain *L.acidophilus* NCDC15 and isolated strains SSR11, SSR14, SSR16. Data are expressed as mean ± SD (n = 3); * $P < 0.05$, *** $P < 0.001$.

6.2.3.2. Autoaggregation assay

From the isolated strains, SSR16 (36.07 ± 0.79 %), was showing highest aggregation percentageas compare to the standard strain LA15 (53.96 ± 0.10 %) after incubated at room temperature for 6 hr. SSR11 (27.55 ± 0.25 %) and SSR14 (31.63 ± 0.10 %) also showed better autoaggregation percentage after incubation as well. This result indicates that the SSR11, SSR14 and SSR16 possess potential ability to adhere to epithelial cells and mucosal surfaces.

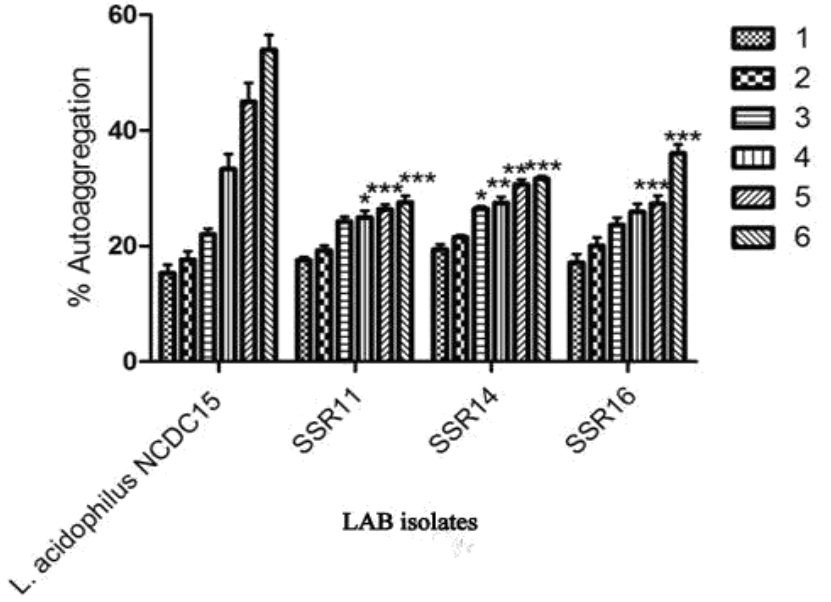


Figure 19 : Autoaggregation of standard strain *L.acidophilus* NCDC15 and isolated strains SSR11, SSR14, SSR16. Data are expressed as mean ± SD (n = 3); * P < 0.05, ** P < 0.01, *** P < 0.001.

6.2.3.3. Coaggregation assay

All tested strains showed some coaggregation properties with pathogens (Fig.20). Among the isolated strains, SSR11, SSR14 and SSR16 showed better coaggregation with *E. coli* as compared to *L. acidophilus* NCDC15 standard strain. SSR16 showed better coaggregation among the isolates.

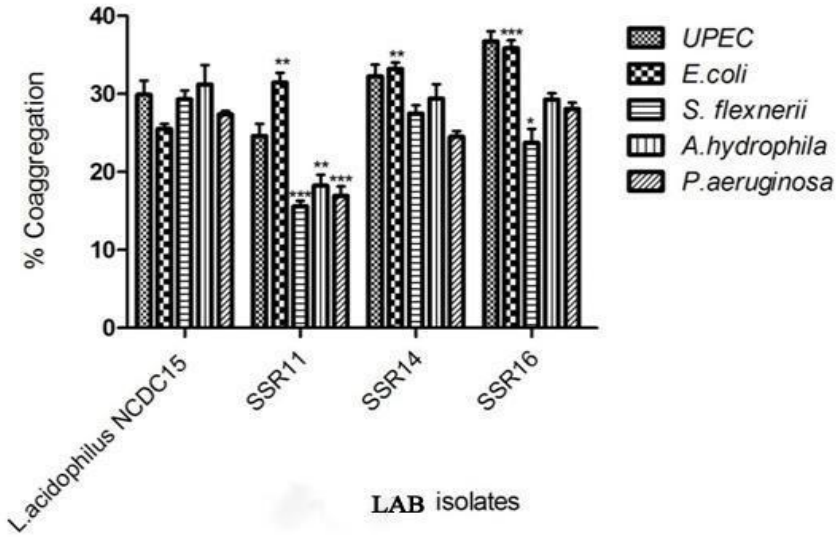


Figure 20 : Coaggregation of standard strain *L.acidophilus* NCDC15 and isolated strains with pathogens SSR11, SSR14, SSR16. Data are expressed as mean ± SD (n = 3); * P < 0.05, ** P < 0.01, *** P < 0.001.

SSR11 showed highest coaggregation property with *E. coli*(31.49%) and lowest with *S. flexneri*(15.62%). SSR 11 showed higher coaggregation property with *E. coli*(31.49%) than compared with *L. acidophilus* NCDC15(25.50%).

SSR 14 showed highest coaggregation property with *E. coli*(33.21%) and lowest with *P. aruginosa*(24.53%). SSR 14 showed higher coaggregation property with *E. coli*(33.21%) and with UPEC(32.29%) than compared with *L. acidophilus* NCDC15 with *E.coli* (25.50%) and with UPEC(29.93%)

SSR16 showed highest coaggregation property with UPEC(36.74%) and lowest with *S. flexneri*(23.75%). SSR 16 showed higher coaggregation property with UPEC(36.74%) ,with *P. aeruginosa*(28.08%) and with *E. coli*(35.87%) than compared to *L. acidophilus* NCDC15which showed UPEC(29.93%) ,with *P.aeruginosa*(27.37%) and with *E. coli*(25.50%).

6.3. Partial purification of antimicrobial protein from CFS of LAB isolates

Total proteins were isolated from cell free supernatant by ammonium sulfate precipitation.

The total protein concentration was estimated by Folin Lowry method (Table 19). Presence of antimicrobial protein in SRN4p was determined by O.D based study of crude protein against *A. hydrophila* (Fig.21) and molecular mass was determined by SDS PAGE (Fig.22). Size exclusion chromatography of SRN4 crude protein (Fig.26) was performed to purify the antimicrobial protein.

6.3.1. Folin Lowry protein estimation of lactobacilli isolates

Table 19: Total protein estimation by Folin Lowry method of standard strain and isolated Strains

Sr. No.	Protein	Concentration (mg/mL)
1	SRN3p	3
2	SRN4p	5

6.3.2. Determination of antimicrobial activity of crude SRN4 protein against *A. hydrophila*

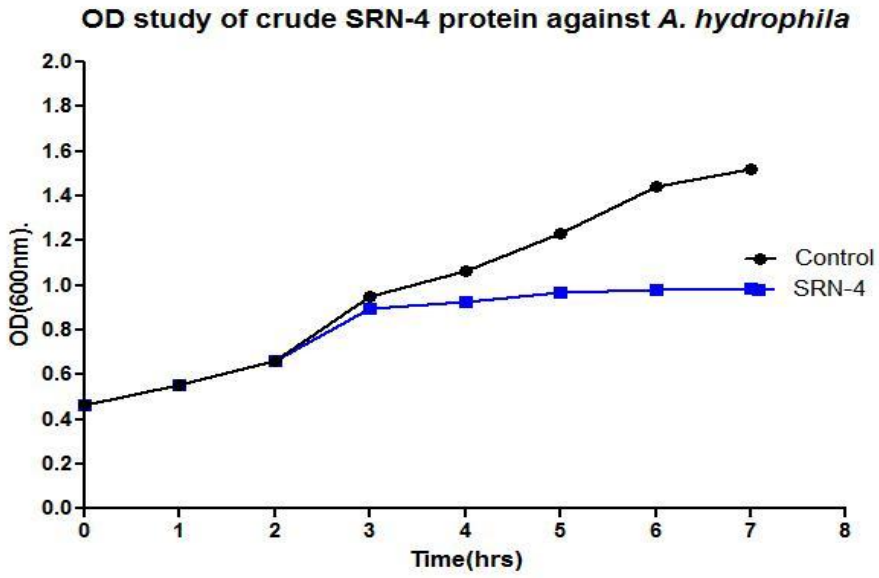


Figure 21: Mode of action of bacteriocin like protein from lactobacilli Isolate SRN4 against *A. hydrophilla*

6.3.3. SDS-PAGE of LAB isolates

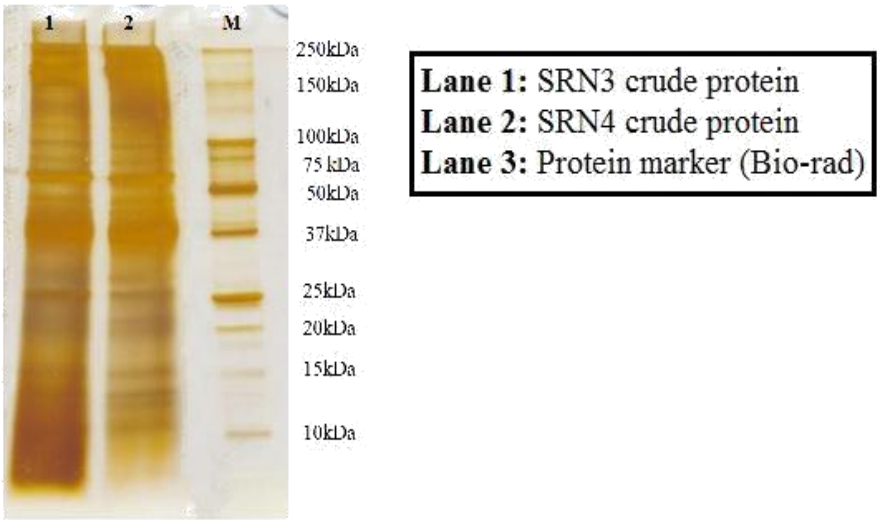


Figure 22: SDS-PAGE of crude protein of SRN3, SRN4 crude protein

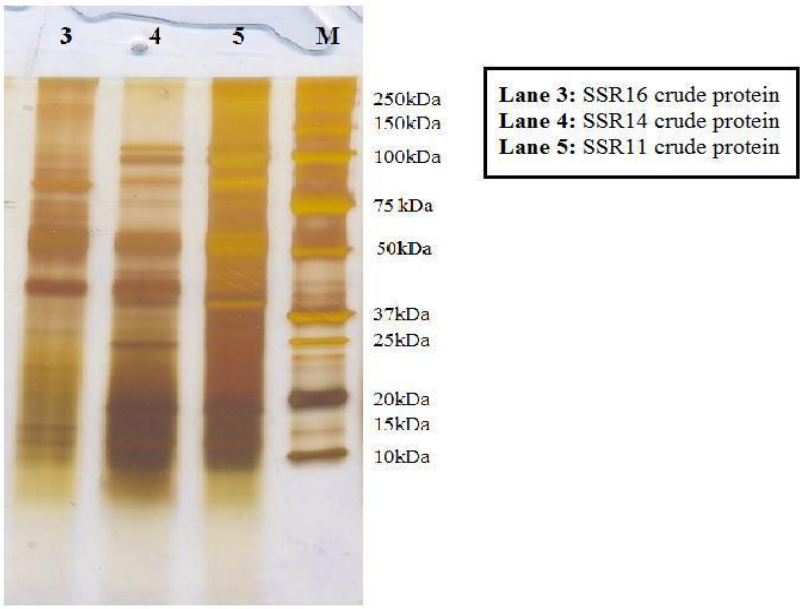


Figure 23: SDS-PAGE of crude protein of SSR16, SSR14 and SSR11 crude protein

6.3.4. RP-HPLC of SRN4 crude protein

The purity of SRN4 crude protein sample was checked by performing RP-HPLC.

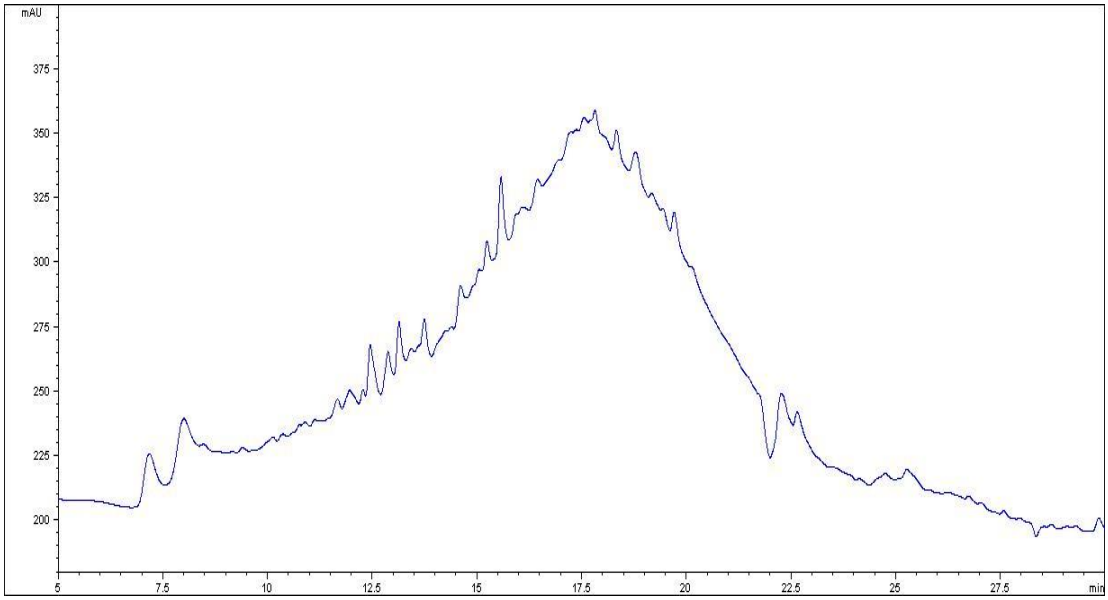


Figure 24: RP-HPLC of SRN 4 crude protein sample

6.3.5. Size exclusion chromatography of SRN4 crude protein

The SRN 4 crude protein was purified by size exclusion chromatography and eluted peak was collected in 24 fraction, named as F1 to F24. The F1-F19 fractionation volume was 0.5 mL and F20-F24 volume was 1mL. The individual fractions would be analysed by RP-HPLC**.**

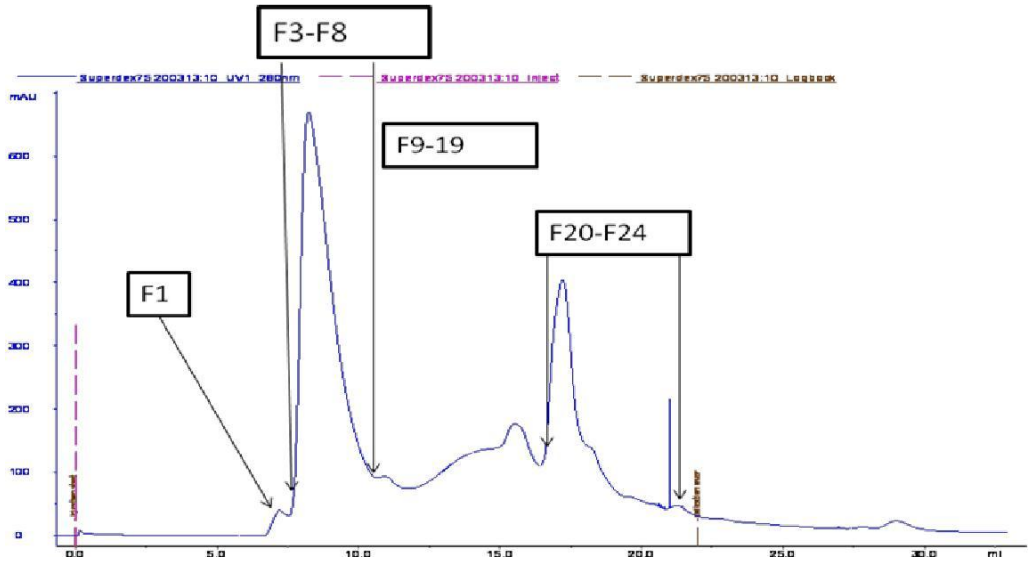


Figure 25: Size exclusion chromatography of SRN 4 crude protein sample

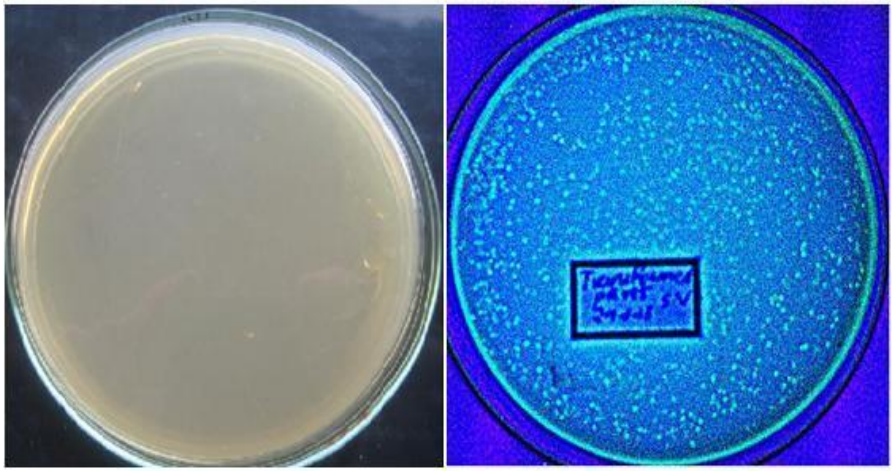
All the 24 fractions will be tested against pathogen, and the fraction giving an inhibitory zone will be further purification of antimicrobial protein by RP-HPLC.

6.4. Genetic modification study

6.4.1. Transformation and plasmid isolation of pRV85

6.4.1.1. Transformation of pRV85 plasmid

The pRV85 vector was transformed in to the *E. coli* BL21 and 280 colonies were obtained after the overnight incubation on LB plates containing 150 µg/mL erythromycin.



(a) Control plate (b) pRV85 transformed plate

Figure 26: Transformation plate of pRV85

6.4.1.2. pRV85 plasmid isolation from transformed *E. coli* BL21

DNA bands were seen in lane 1, 2, 3, 4, 5 and 6 which resembled the size of pRV85 i.e. 6.2 Kb compared to the marker on 1% agarose gel.

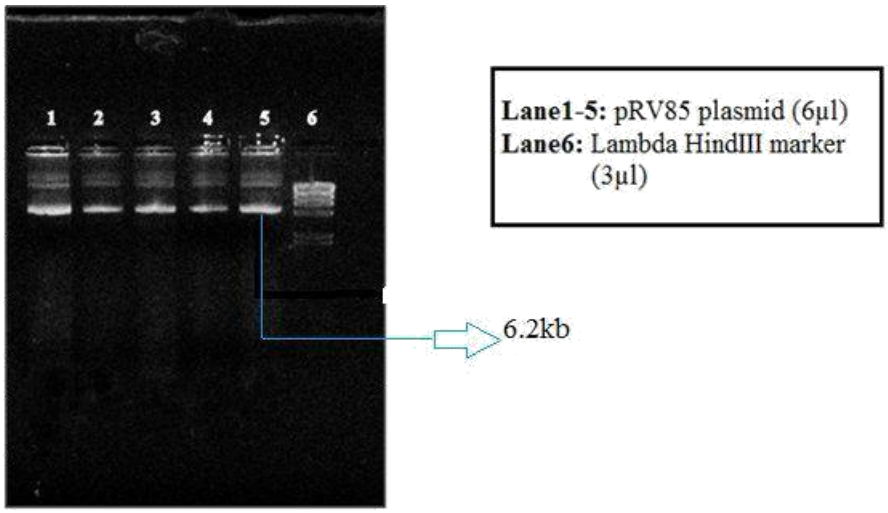
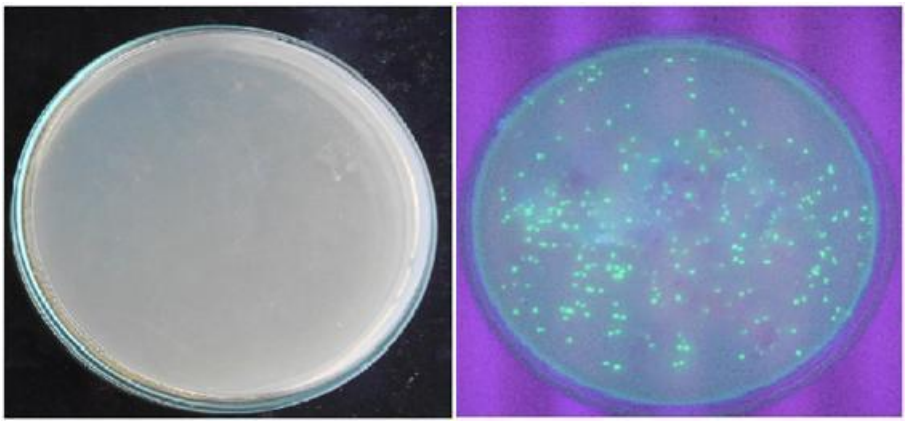


Figure 27: Gel image of pRV85 isolated plasmid

6.4.2. Transformation, plasmid isolation and RE digestion of pRV86 plasmid

The pRV86 vector was transformed in to the *E. coli* BL21 and 130 colonies were obtained after the overnight incubation on LB plates containing 150 µg/mL erythromycin.

6.4.2.1. Transformation of pRV86 plasmid



(a) Control plate (b) pRV86 transformed plate

Figure 28: Tranformation plate of pRV86

6.4.2.2. pRV86 plasmid isolation from transformed *E. coli* BL21

DNA bands were seen in lane 1, 2, 3, 4, 5 and 6 which resembled the size of pRV86 i.e. 6.3 Kb compared to the marker on 1% agarose gel.

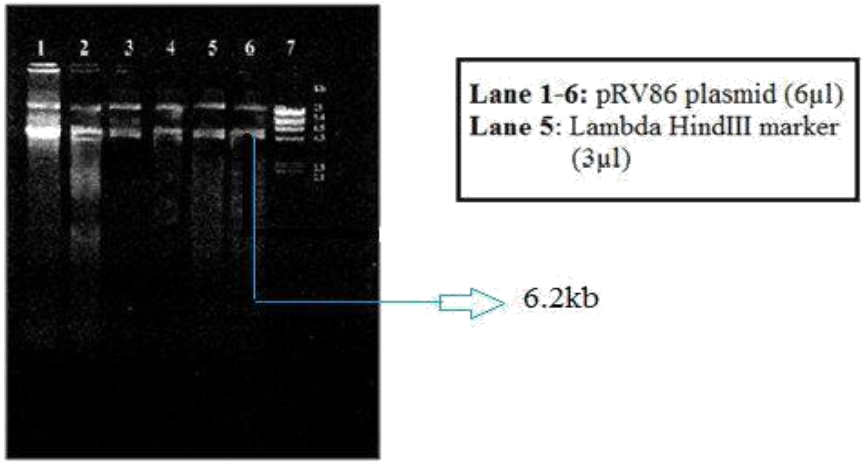


Figure 29: Gel image of isolated plasmid pRV86

6.4.2.3. Restriction Digestion of pRV86

pRV86 plasmid was digested with the enzymes *Sph*I and a linear fragment of the vector was obtained.

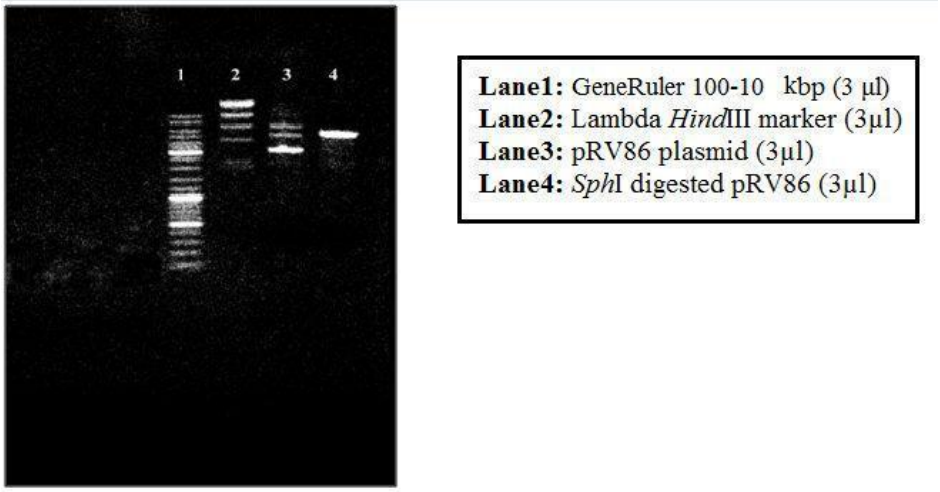
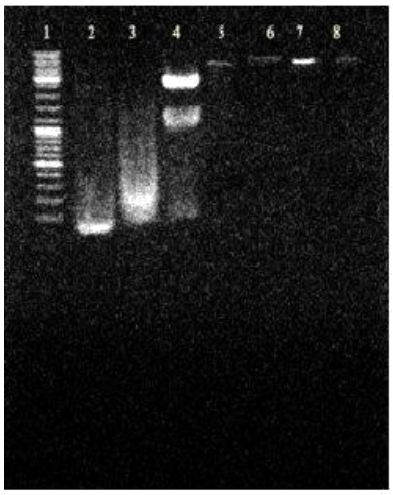


Figure 30: Gel image of restriction digestion of pRV86 with *Sph*I.

6.4.2.4. Gel extraction of digested pRV86 plasmid

Linear fragment of *Sph*I digested pRV86 plasmid was extracted from the agarose gel using the Sure Extraction/ PCR Clean up Kit.



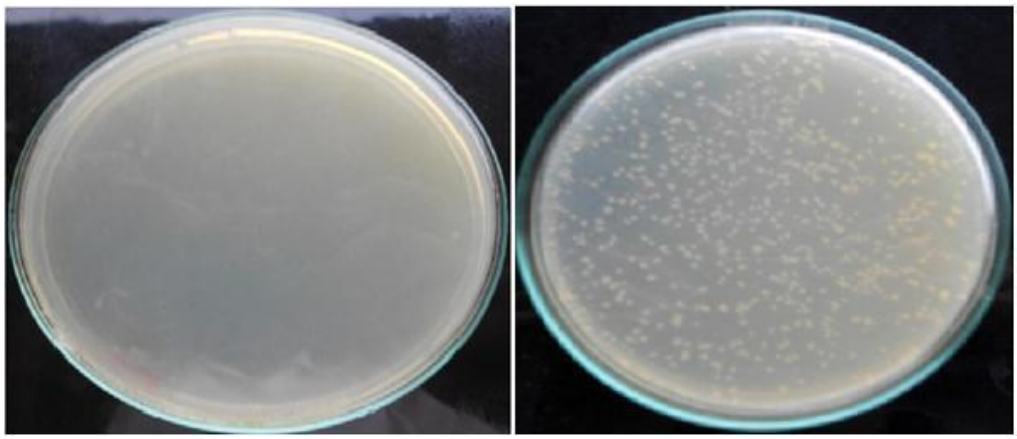
Lane1: GeneRuler 100-10 kbp (3µl)
Lane7: *Sph*I digested pRV86 plasmid (3µl)

Figure 31: Gel extraction of restricted pRV86

6.4.3. Transformation, isolation and RE digestion of pSLP111.3 plasmid

The pSLP111.3 vector was transformed in to the *E. coli* BL21 and colonies were obtained after the overnight incubation on LB plates containing 10 µg/mL chloramphenicol.

6.4.3.1. Transformation of pSLP111.3 plasmid



(a) Control plate (b) pSLP111.3 transformed plate

Figure 32: Tranformation plate of pSLP111.3

6.4.3.2. pSLP111.3 plasmid isolation from transformed *E. coli* BL21

DNA bands were seen in lane 1, 3, 4 and 5 which resembled the size of pSLP111.3 i.e. 4.1 Kb compared to the marker on 1% agarose gel.

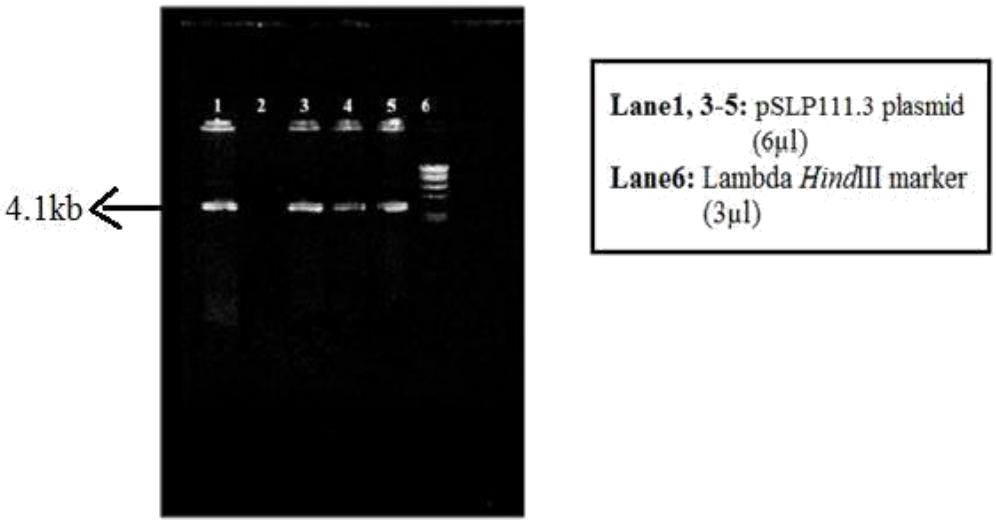


Figure 33: Gel image of pSLP111.3 isolated plasmid

6.4.3.3. Restriction Digestion of pSLP111.3

pSLP111.3 plasmid was digested with the enzymes *Nhe*I and *Hind*III and three fragments of the size 2.7 kb and 900bp and 503 bp were obtained respectively on 1.2% agarose gel.

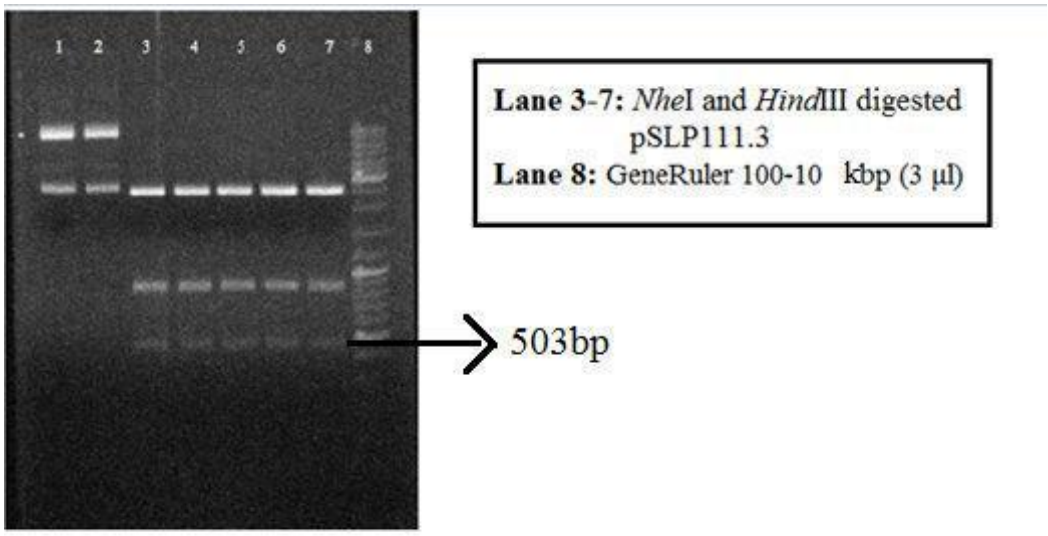


Figure 34: Gel image of restriction digestion of pSLP113.1 plasmid

6.4.3.4. Gel extraction of digested pSLP111.3 plasmid

Fragment of the size 500bp from digested pSLP111.3 plasmid was extracted from the agarose gel using the Sure Extraction/ PCR Clean up Kit.

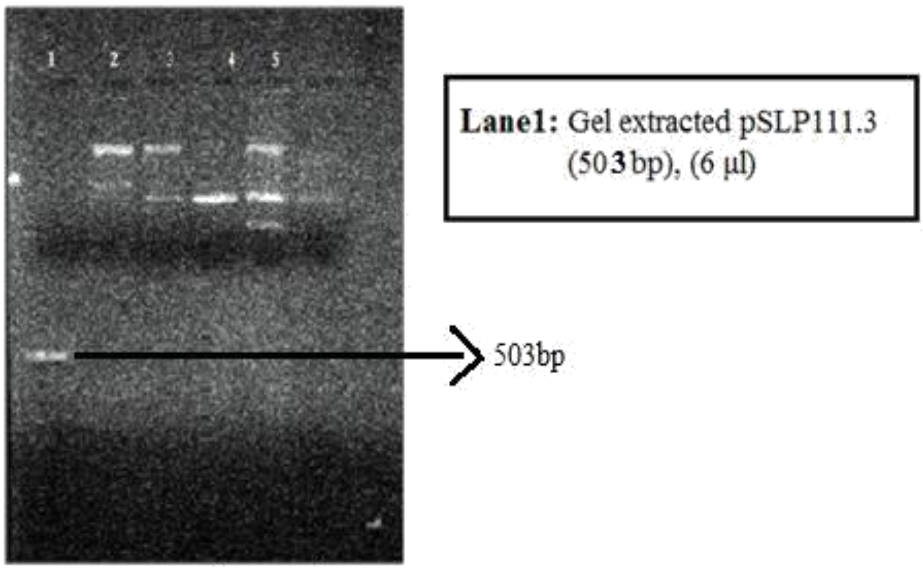


Figure 35: Gel extraction from restricted pSLP111.3

6.4.4. Plasmid isolation and PCR of pColE2-P9 plasmid

6.4.4.1. Plasmid isolation of pColE2-P9 plasmid

pColE2-P9 was isolated from the *E.coli* BL21, DNA bands were seen in lanes 5-8 which exactly resembled the size of pColE2-P9 i.e. 7.2 Kb compared to the marker on 1% agarose gel.

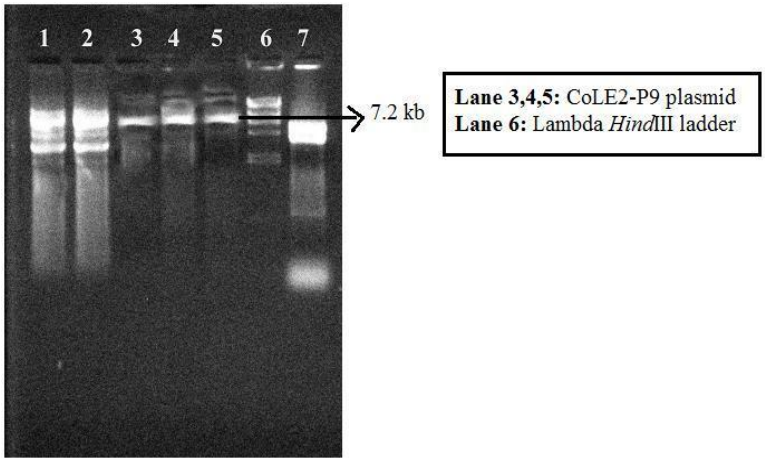
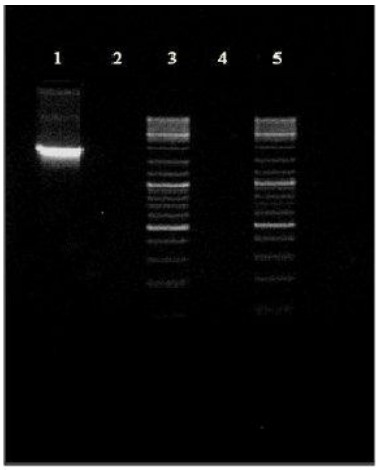


Figure 36: Gel image of pColE2-P9 plasmid.

6.4.4.1. Polymerase chain reaction (PCR) of pColE2-P9 plasmid

The amplification of the Colicin E2 using its primers has been successfully obtained and the band of 2039 bp of the amplicon can be seen in the (1.2 %) agarose gel.

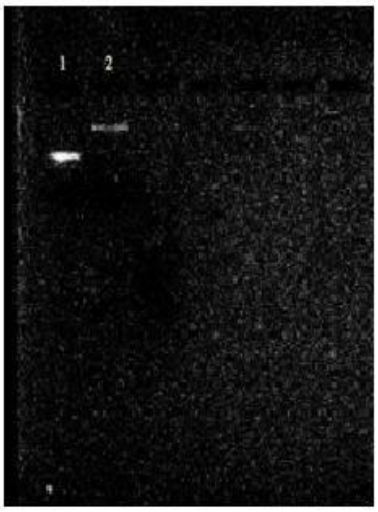


Lane 1: PCR amplified product of ColE2 gene
Lane 3, 5: GeneRuler 100-10 kbp (3µl)

Figure 37: Gel image showing the band of amplified fragment of Colicin E2 gene

6.4.4.2. Gel extraction from of colE2 amplicon:

Fragment of the size 2Kb from amplified pCOLEp9 plasmid was extracted from the agarose gel using the Sure Extraction/ PCR Clean up Kit.



Lane 1: PCR amplified product of colicin E2 gene
Lane 2: *Sph*I digested pRV85

Figure 38: Gel extraction from of colE2 amplicon

6.4.5. Ligation

6.4.5.1. Ligation of pRV86 with pColE2

Before performing ligation *Sph*I digested pRV86 was blunt ended and treated with shrimp alkaline phosphatase enzyme to avoid self ligtion of vector. 2Kb amplicon of colE2 was

ligated to pRV86 using Rapid DNA Ligation Kit. Blunt end ligation strategy was used to clone the colicin gene. 50 ng of pRV86 vector and 200 ng of pColE2 were used for ligation.

6.4.5.2. Ligation of pRV86 with *slp*A

Before performing ligation *Sph*I digested pRV86 was blunt ended and treated with shrimp alkaline phosphatase enzyme to avoid self ligtion of vector. 500 Kb insert of pSLP111.3 was ligated to pRV86 using Rapid DNA Ligation Kit. Blunt end ligation strategy was used to clone the *slp*A gene. 50 ng of pRV86 vector and 200 ng of *slp*A were used for ligation.

Transformation of ligated products into *E. coli* Dh5α strains is in process.

6.4.6. Electroporation of pRV85 in SRN3

100 ng of pRV85 was electroporated into competent SRN3 isolate, spreaded on MRS plates containing erythromycin marker, and incubated at 30 ˚C for 48 hours.The colonies obtained are shown in (Fig.39).

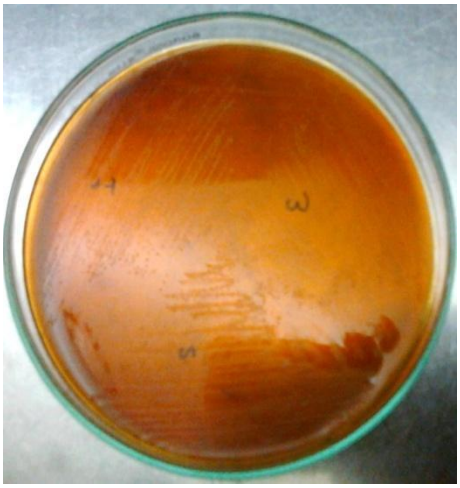


Figure 39: Transformed SRN3 with pRV85 plasmid

7.DISCUSSION

It has been observed that many pathogenic bacterial species cause severe outbreak of infectious diseases in animals and humans. Use of antibiotics to control these agents has led to problems of drug resistance and brings important changes in the commensal microbiota of the systems and surrounding environment. Therefore, it is important to seek and combat these pathogens with the development of alternative methods.

As an alternative strategy to these antimicrobial compounds, the prophylactic use of beneficial bacteria (probiotics) has emerged to improve health and provide nutrition (57). LAB species are commonly found among the resident microbiota of the gastrointestinal tract and genitourinary tract of humans and animals (81,82) Most of the probiotics have been isolated from various sources like gut microbiota, vaginal sources, oral cavity, yoghurt and vegetable etc. Among these, gut microbiota plays an important role in maintaining human health, not only due to its participation in the digestion process, but also for the function it plays in the development of the gut and the immune system (83).

Because of their history of safe use and their natural presence in the intestinal tract, commensal lactobacilli offer considerable potential as probiotics amongst all lactic acid bacteria group. In this study lactobacilli were isolated from rat fecal sample and characterized for their probiotics properties. Also these isolates were further genetically engineered for enhancement of its efficiency and colonization in the gastrointestinal tract.

Lactic acid bacteria were recently described as part of the normal microbiota in freshwater fish. Probiotics is a big business today in Indian aquaculture; it is worth $109 million, and most supplies are imported. Indian fish pathologists are looking at probiotics as a potentially useful disease prevention measure in aquatic farms, and active research is continuing in this regard (84). However, the efficiency of probiotics isolates from tropical freshwater species is less studied and needs further exploration.

Himatnagar is a tropical district situated at the southern part of Gujarat (N 23° 34' 47", W 72° 57' 47") and is well known for its freshwater fish production and its supply to central part of Gujarat.

In this study, LAB were isolated from intestinal tract of fish Rohu (Fresh water fish, *Labeo rohita*) from the above cited region. Normal microflora in the intestinal tract of fishes inhabiting fresh water reservoirs includes LAB. The number of bacteria depends on the animal species, the composition of food, the age and the season. Various species of lactobacilli are present in relatively high number in the intestines of freshwater fish in warm season but low in number in cold season (85) Therefore, in this study enterococci were abundantly present as the fish was brought in cold season. The isolated coccoid LAB were investigated for their antagonistic activity against various pathogens like *A. hydrophilla, S. flexnerii* MTCC1457, *E. coli* MTCC729 (uropathogenic), *P. aeruginosa* MTCC1688 and *E. coli* MTCC 443which were the major cause of mortality in fish hatcheries as well as cause of other infectious disease.

In the present investigation we had identified four lactobacilli isolates (SRN3 andSRN4) (SSR11, SSR14 and SSR16) from gut of *Labeo rohita* as potent probiotics. To be considered as probiotics, these bacteria should become a part of the normal microbial flora in the intestine, survive the gastrointestinal passage, and be able to adhere and colonize the intestinal tract (38). The isolated lactobacilli (SRN3 and SRN4) were showed intrinsic vancomycin resistance and showed yellow colonies on MRS+BCP plates resulting production of lactic acid, whereas fish isolated LAB (SSR11, SSR14 and SSR16) were sensitive to vancomycin and also confirmed by production of lactic acid.

All the five isolates showed resistance to gastric acid (pH 2.5) and may be able to survive passage through the digestive system that has specific condition such as the low pH of the stomach. The isolated lactobacilli showed an amplicon size of 232bp by PCR amplification using *Lactobacillus* specific 16S rRNA primers (LactoF and Lacto R), which shows specificity against $V_{2.1}$, $V_{2.2}$ and V_3 regions of 16S rRNA. While the LAB isolates from fish were not amplified with universal *Lactobacillus* primers and neither with the *Enterococcus* virulence gene (*gelE*L and *gelE*R). As the morphologyof isolated LAB had come out to be coccoid in shape, so it can be prorposed to belong to *Enterococcus* species, but strain identification through PCR amplification with 16SrRNA specific primers is under process to confirm it to be a *Enterococcus*, *Streptococcus*, *Pediococcus* etc.

Antimicrobial spectra of the isolated probiotics used in this study were also tested against different pathogens mentioned earlier. The ability to inhibit the growth of harmful bacteria is also considered as a desirable feature for probiotic bacteria. Previous studies have demonstrated diverse growth inhibition of different pathogens by many lactobacilli strains originating from various foods (86) or humans (87) and animals (88)

Antibiotic susceptibility is an important aspect for an organism to be a potential probiotics candidate. In order to discard the presence of transferable antibiotic resistance genes in any of the candidate probiotics strains, antimicrobial resistance profile was assessed. Here all the isolated strains of *Lactobacillus* (SRN3 and SRN4) and Lab isolates from fish (SSR11, SSR14 and SSR16) were found to be sensitive against various antibiotics but lactobacilli were intrinsic resistance to vancomycin.

The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property for probiotics. It prevents their immediate elimination by peristalsis, and provides a competitive benefit in the ecosystem . Bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesins (usually proteins) and complementary receptors . The surface components (proteins and lipids) and hydrophobicity of the cell surface are important for aggregation. Physicochemical characteristics of the cell surface such as hydrophobicity may affect autoaggregation and adhesion of bacteria to different surfaces (89).

The isolated *Lactobacillus* SRN4 showed higher hydrophobicity with toluene and xylene as compared to other isolated strain, SRN3. Both the strains SRN3 and SRN4 showed higher amount of hydrophobicity percentage i.e 48.03±0.89, 51.71±0.92 for xylene and 44.85±0.83, 55.82±0.68 for toluene accordingly as compared to standard strain *L. acidophilus* NCDC15 which showed 37.04±0.43 percent hydrophobicity to xylene and 55.1±0.76 percent hydrophobicity to toluene. Among them SRN4 showed highest amount of % hydrophobicity for both xylene and toluene.

Isolated strains ofLAB SSR11, SSR14 and SSR16 showed lower amount of hydrophobicity percentage i.e 10.54±0.89, 13.59±0.92, 25.88±0.78 for xylene and

17.26±0.83, 25.33±0.68, 25.41±0.98 for toluene accordingly as compared to standard strain *L. acidophilus* NCDC15 which showed 37.04±0.43 percent hydrophobicity to xylene and 55.1±0.76 percent hydrophobicity to toluene. SSR16 showed higher amount of % hydrophobicity for both xylene and toluene among all the three isolates.

Autoaggregation correlates with adhesion, which is a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens. In the present study the autoaggregation ability of isolates were considerably increased with increased incubation period. SRN3 and SRN4 both the isolated strains were showing higher *autoaggregation* percentage as compare to the standard strain *L. acidophilus* NCDC15 after incubation at 37°C for 6 hours. SRN3 and SRN4 strains *were* showing 62.43±0.45 and 62.92±0.98 percentage of autoaggregation which is higher than the standard strain *L. acidophilus* NCDC15 (53.96±0.99). This result indicates that the SRN3 and SRN4 possess potential ability to adhere to epithelial cells and mucosal surfaces.

Among the probiotic LAB isolates from fish, SSR16 (36.07 ± 0.79 %), was showing highest aggregation percentage as compare to the standard strain *L. acidophilus* NCDC15 (53.96 ± 0.10 %) after incubated at room temperature for 6 hr. SSR11 (27.55 ± 0.25 %) and SSR14 (31.63 ± 0.10 %) also showed better autoaggregation percentage after incubation as well. Bacterial aggregation between microorganisms of the same strain (autoaggregation) or between genetically different strains (coaggregation) is of significant importance in numerous ecological niches, especially in the human gut, where probiotics are to be active.

In order to evaluate cell–cell adherence, a coaggregation assay was taken and established coaggregation between selected strains and pathogens. It has been suggested that inhibitors or bacteriocin producing lactic acid bacteria, which coaggregate with pathogens, may contribute to host defence against infection. Coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms (4). All tested strains showed some coaggregation properties with pathogens. Among theisolated strains, SRN3 and SRN4 showed better coaggregation with UPEC, *P. aeruginosa, A. hydrophila,* and *E. coli* as compared to *L. acidophilus* NCDC15 standard strain.

SRN 3 and SRN 4 showed highest coaggregation with *P. aeruginosa* (51.33%), and *E. coli* (57.84%) respectively. Both these strains showed lowest coaggregation potentials with UPEC *i.e.*, 37.48% and 38.36 respectively, while moderate coaggregation was observed with *S.* Typhimurium (42.06% and 46.76% respectively). Both these strains showed better coaggregation efficiency compared to CS7, our lab isolate (1).

All tested isolates of LAB from fish intestine, showed some coaggregation properties with pathogens. Among the isolated strains, SSR11, SSR14 and SSR16 showed better coaggregation with *E. coli* as compared to *L. acidophilus* NCDC15 standard strain. SSR16 showed better coaggregation among the isolates.

SSR16 showed highest coaggregation property with UPEC(36.74%) and lowest with *S. flexneri*(23.75%). SSR16 showed higher coaggregation property with UPEC(36.74%) ,with *P. aeruginosa*(28.08%) and with *E. coli*(35.87%) than compared to *L. acidophilus* NCDC15which showed UPEC(29.93%), with *P. aeruginosa*(27.37%) and with *E. coli*(25.50%)

To determine the presence of antimicrobial protein in CFS of SRN4 strain, crude protein was isolated by ammonium sulfate precipitation method and its activity against *A. hydrophila* was checked. The isolated crude protein showed inhibitory property against pathogen. SDS-PAGE, RP-HPLCand size exclusion chromatography were performed to purify the antimicrobial protein and 24 fractions were collected. The spot test of each fraction will be done against different pathogens and the fraction showing zone of inhibition will be purified by performing RP-HPLC.

Genetic modification of probiotic bacteria is needed to improve the effectiveness of its existing properties or to add new beneficial activities (e.g. vaccine presentation). Hence there is an interest in development of genetic tools for efficient and controllable gene expression in lactobacilli.

It has been reported that lactobacilli have great potential as delivery vehicles for interesting proteins, such as antigens, antibodies and growth factors (64,13 Pavan et al., 2000; Kruger et al., 2002; Scheppler et al., 2002). Lactobacilli are currently under investigation for use in

active vaccination, passive vaccination and tolerance induction. As such, these bacteria are consumed in large amounts by humans and have been so for thousands of years without causing any known health problems. This makes them attractive candidates for the development of safe (oral) vaccines.

The aim of the present study was to develop an efficient protein-secretion system using recombinant lactobacilli for various applications such as live delivery of biotherapeutics. The yield of secreted protein is preferred to be high for better effects. Hence, strong promoters should be used for the expression of heterologous genes. It is also known that specific amino acid sequences flanking signal peptides can enhance the efficiency of protein secretion. Using these strategies, several highly efficient secretion systems have been developed in LAB.

For the construction of expression vectors, the *Lactobacillus lactis* secreting biologically active interleukin 10 (IL-10) was established for the treatment of inflammatory bowel diseases in a murine model while recombinant *L. lactis* and *Lactobacillus plantarum* that secrete microbiocidal cyanovirin-N were constructed and were capable of neutralizing the infectivity of HIV-1. (6) *in vitro*. These studies suggested that protein-secreting systems in LAB could be useful and offer a promising strategy for medical applications in the future.

In present study various *E. coli-Lactobacillus* shuttle vectors were used (Table 6) to develop genetic engineered lactobacilli probiotics. For the extracellular expression of protein, signal peptide is required. Therefore a secretion vector pSLP111.3 was used which was having *slp*A signal peptide, *pxyl*A promoter and cell wall adhesive anchor protein with multiple cloning sites for insertion of gene of interest.

To achieve these objectives various strategies were adopted. The colicinE2 gene was isolated from plasmid pColE2-P9, amplified, and ligated with pRV86 shuttle vector which was earlier digested with restriction endonuclease *Sph*I, blunt end filled and treated with shrimp alkaline phosphatase to dephosphorylate 5’ to 3 end of linear vector and remove self ligation as well.

On the other hand *slp*A signal sequence was extracted from pSLP111.3 by digestion with *Nhe*I and *Hind*III to get 500bp fragments, blunt ended and ligated with *Sph*I digested blunt ended pRV86.

The ligated products were transformed in *E. coli*Dh5α competent cells, but the transformed colonies were not obtained on L.B agar plates containing ampicillin (100µg/mL) as selective marker. The possible reasons may be, (i) The insert and vector may not have blunted properly due to which ligation could not result (ii) After blunt end and alkaline phosphatase treatment the vector was not purified by phenol chloroform treatment, as a result presence of unwanted salts and other debries of denatured enzyme may result in ligation problems. (iii) Due to codon biasness.

pRV85 shuttle expression vector having gfp gene was electroporated in SRN3 lactobacilli isolate and spreaded on MRS agar containin erythromycin as selective marker. After 48 hours of incubation period small colonies of lactobacilli were obtained. Further conformation of transformed pRV85 is remaining.

8.CONCLUSION

SRN3 and SRN4 strains of lactobacilli isolated from fecal sample of male wistar rat were found to posses potential probiotic and antimicrobial properties against various pathogens. Further, genetic modification of these isolates makes them more efficient in the field of probiotic research. The isolated lactobacilli strain SRN4 showed almost similar probiotics properties to SRN3.

SSR16 strain of LAB isolated from intestine of fish (Rohu) showed better probiotics and antimicrobial properties as compared to SSR11 and SSR14.

The crude protein of SRN4 isolate showed antimicrobial activity against *A. hydrophila.* Purification of antimicrobial protein needs to be done.

Transformation of constructs into *E. coli*Dh5α resulted into no growth of trasformants.

Attemps are being made to make genetically modified construct of lab islolated probiotic strain of lactobacilli.

The constructed vector pRV86-slpA would be meeting the adequate requirement of novel shuttle secretion vector that has enormous uses like genetic modification of probiotic strain, heterologus protein expression system , as vaccine delivery system, which has numerous medical and commercial advantages.

The constructed vector pRV86-ColE2 having colicin gene insert can be used in treatment of urinary tract infection against uropathogenic *E. coli*.

Lactobacilli isolate SRN3 was electroporated with pRV85 plasmid in order to obtain green fluorescence phenotype, can be used in monitoring colonization of probiotic SRN3 with GI tract epithelial cells.

9.APPENDIX

APPENDIX – I (Media)

- LB Medium (Luria-Bertani HiVeg Broth):

Ingredients	**(g/L)**
HiVeg hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00

Shake until the solutes have dissolved. pH was adjusted to 7.5 with 5 N NaOH. Volume of solution was adjusted to 1 liter with deionized H_2O and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle. LB agar plates were made by adding 2.5% agar-agar powder to LB medium.

- MRS Medium:

Ingredients	**(g/L)**
HiVeg peptone No.3	10.00
HiVeg extract	10.00
Yeast extract	5.00
Dextrose	20.00
Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Mangnese sulphate	0.05
Dipotassium phosphate	2.00

Shake until the solutes have dissolved. pH was adjusted to 6.5 with 5 N NaOH. Volume of solution was adjusted to 1 liter with deionized H_2O and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle. MRS agar plates were made by adding 2.5% agar-agar powder to MRS medium.

- Nutrient Medium:

Ingredients	**(g/L)**
HiVeg peptone	5.00
HiVeg extract	1.50
Yeast extract	1.50
Sodium chloride	5.00

Shake until the solutes have dissolved. pH was adjusted to 7.4 with 5 N NaOH. Volume of solution was adjusted to 1 liter with deionized H_2O and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle. NB agar plates were made by adding 2.5% agar-agar powder to NB medium.

APPENDIX – II: (Buffers and Solutions)

- Alkaline Lysis Solution I (plasmid preparation)

50mM glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Solution was prepared from standard stocks in batches of ~ 100 mL and autoclaved for 15 minutes at 15 psi on liquid cycle, and stored at 4 ^{o}C.

- Alkaline Lysis Solution II (plasmid preparation)

0.2 N NaOH (freshly diluted from a 10 N stock)

1 % (w/v) SDS

Solution II was prepared freshly and used at room temperature.

- Alkaline Lysis Solution III (plasmid preparation)

5 M potassium acetate : 60.0 mL
Glacial acetic acid :11.5 mL
H_2O : 28.5 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate and solution was stored at 4^0C

- TAE Buffer (50X)

Stock Solution/Litre
242 g of Tris base
57.1 mL of glacial acetic acid
100 mL of 0.5 M EDTA (pH 8.0)
Dilute the concentrated stock buffer just before use.

- 10X T.E buffer (pH 8.0)

100 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)
Solution was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle and buffer was stored at room temperature

.

- Glycerol (10% v/v)

Dilute 1 volume of glycerol in 9 volumes of sterile pure H_2O. Solution was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle and stored at $4\ ^oC$.

- Glycerol (50% v/v)

Dilute 5 volume of glycerol in 5 volumes of sterile pure H_2O. Solution was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle and stored at room temperature.

- 0.1M $CaCl_2$

0.3 g CaCl2 .$2H_2O$ was dissolved in 20 mL of deionized H_2O.

- Phosphate Buffer Saline (pH 7.0)

For 500 mL,
NaCl – 4 g

KH_2PO4 – 0.17 g

K_2HPO4 – 0.605 g

Dissolved in 300 mL distilled water and pH was to 7.0. Final volume was made up to 500 mL.

APPENDIX –III : (Chemicals and Reagents)

- Ethidium Bromide (10mg/mL)

  10 mg Ethidium Bromide was added to 1 mL H_2O and stirred on a magnetic stirrer for several hours to ensure that dye had dissolved.

- Antibiotics

  Erythromycin (Himedia), Ampicilin (Himedia), Chlormphenicol (Himedia), Streptomycin (Himedia),

- Stains

  Gram staining reagents
  Crystal violet
  Gram's Iodine
  Destainer
  Saffrenine

- Chemicals
  - Agarose (Himedia)
  - Ammonium sulfate
  - Ethylene diamine tetra acetic acid disodium EDTA) (Himedia)
  - Sodium acetate (Merck)
  - SDS (Sodium Dodecyl Sulphate) (Sigma)
  - Manganese chloride (S.D. Fine chemicals)
  - Calcium chloride (S.D. Fine chemicals)
  - Tris hydroxyl methyl amino methane (Himedia)
  - Ethidium bromide (Merck), Magnesium chloride (S. D. Fine chemicals)

- Glucose (Himedia), Sodium hydroxide pellets (Merck)
- Potassium acetate (Himedia)
- Glacial acetic acid (Himedia)

APPENDIX – IV : (protocols)

- Plasmid Preparation by Alkaline Lysis Method (MINIPREP)

The plasmid preparation was done by alkaline lysis method as per the protocol in (80).

- Preparation of cells

A single colony of transformed bacteria was inoculated in 2mL rich medium (LB) containing appropriate antibiotic. Culture was incubated overnight at 37^{O}C with vigorous shaking. 1.5 mL culture was taken into microfuge tube and centrifuged at maximum speed for 30 sec. at 4^{O}C. Medium was removed by aspiration, leaving the bacterial pellet as dry as possible.

- Lysis of cells

Bacterial pellet was resuspended in 100µl of ice-cold solution I by vigorous vortexing.

200µl of freshly prepared alkaline lysis solution II was added to each bacterial suspension. Tube was closed tightly and content was mixed by inverting tube rapidly 5 times and stored on ice for 10 min. 150µl of ice-cold alkaline lysis solution III. Tube was closed and dispersed alkaline solution III through the viscous bacterial lysate and inverted several times. Tube was stored on ice for 3-5 min. Bacterial lysate was centrifuged at maximum speed for 5 min at 4^{O}C in a microfuge tube. Supernatant was transferred to a new microfuge tube. Equal volume of phenol: chloroform was added. Organic and aqueous layer was mixed by vortexing and then centrifuged at maximum speed for 2 min. at 4^{O}C in a microfuge. Supernatant was transferred to a new tube.

- Recovery of plasmid

Nucleic acid was precipitated from the supernatant by adding 2 volumes of ethanol at room temperature. Solution was mixed through vortexing and allowed to stand at R.T for 2 min. Precipitated nucleic acid was collected by centrifugation at maxi. Speed for 5 min at 4^{O}C in

a microfuge. Supernatant was removed by gentle aspiration. Tube was allowed to stand in an inverted position and all fluid was allowed to drain away. 1 mL of 70% ethanol was added to the pellet and inverted the closed tube several times. DNA was recovered through centrifugation at maxi. Speed for 2 min. at 4^{o}C in microfuge. Supernatant was removed through gentle aspiration. Ethanol beads were removed from sides of tubes. Open tube was stored at R.T until all ethanol had evaporated. Nucleic acids were dissolved in 50 µl T.E (pH- 8.0) containing 20µg /mL DNase- free RNase. DNA solution was stored at -20^{o}C.

- Plasmid isolation by GeneJet TM Plasmid Miniprep Kit

A single bacterial colony from a freshly streaked selective plate was inoculated in 10 mL LB medium supplemented with appropriate selection antibiotic. This was incubated for 12-16 hours at 37^{o}C with vigorous agitation. For high copy number plasmids, 5 mL of bacterial culture was harvested while for low copy number plasmids, 10 mL of culture was harvested. The culture was harvested by centrifugation at 8000 rpm in a microfuge for 2 minutes at room temperature. The supernatant was decanted removing all the remaining medium.The pelleted cells were resuspended in 250 µl resuspension solution by vortexing. 250 µl lysis solution was added and mixed thoroughly. 350 µl neutralization solution was added and mixed by inverting.

Centrifugation was done for 5 minutes to pellet cell debris and chromosomal DNA. The supernatant was transferred to spin column. The column was centrifuged for one minute and the flow through was discarded. 500 µl of wash solution was added to spin column and it was centrifuged for 1 minute and the flow through was discarded. This step was repeated once. The flow-through was discarded and column was centrifuged for additional 1 minute to remove residual wash solution. The spin column was transferred to a 1.5 mL microfuge tube. 50 µl of elution buffer was added to the center of the spin column membrane to elute the plasmid DNA. This was incubated for 2 minutes at room temperature and centrifuged for 2 minutes. The column was discarded and the purified plasmid DNA was stored at -20^{o}C.

- DNA Extraction from Agarose gels (Sure Extraction/PCR Clean Up Kit)

DNA fragment was excised from an agarose gel using sterile scalpel precisely. Gel slice was weighed and transferred to a clean 1.5 mL microcentrifuge tube. For each 100mg of agarose gel 200 µl Buffer SET was added. Sample was incubated for 5- 10 min at 50^{o}C and pulse vortexing was done every 2-3 min until gel slice dissolved completely. Sample was loaded

onto the Sure Extract Spin Gel extraction column placed in a collection tube (2 mL) and centrifuged for 1 min at 11,000 g. Flow- through was discarded and column was placed back into the collection tube. 700 µl Buffer SET3 was added to the Sure Extract Spin Gel extraction column and centrifuged for 1 min at 11,000g. The flow-through was discarded and the column was placed back into the collection tube. Additional centrifugation for 2 min at 11,000 g was done to remove the traces of SET3 buffer. Flow through was discarded. 15-50 µl of SEB buffer was added to the spin column placed in a 1.5 mL eppendorf tube and incubated for 1 min at room temperature followed by centrifugation at 11,000g for 1 min.

Transformation of Plasmid DNA into *E.coli* using calcium chloride method

Transformation of plasmid DNA into *E.coli* was done by calcium chloride method as per the protocol in (80).

Preparation of competent cells

A single bacterial colony was picked that was incubated for 16-20 hours and transferred into 100mL LB broth in a 1- liter flask. Culture was incubated for 3 hours at 37^{o}C with vigorous agitation, monitoring growth of culture. For efficient transformation, it is essential that the number of viable cells not exceed 108 cells/mL, which for most *E.coli* strains was equivalent to an $O.D_{600}$ of Bacterial cells were transferred to a sterile, disposable, ice-cold 50-mL polypropylene tube. Culture was cooled at 0 ^{o}C by storing tubes on ice for 10 minutes. Cells were recovered by Centrifugation at 2700g (4100 rpm) for 10 minutes at 4^{0}C. Medium was decanted from cell pellets and Tubes were allowed to stand in inverted position on a pad of towel for 1 minute to allow all traces of media to drain away. Each pellet was resuspended by swirling or gentle vortexing in 30 mL of ice-cold $MgCl_2 – CaCl_2$ solution (80mM $MgCl_2$, 20mM $CaCl_2$). Cells were recovered by Centrifugation at 2700g (4100 rpm) for 10 minutes at 4^{0}C. Medium was decanted from cell pellets and Tubes were allowed to stand in inverted position on a pad of towel for 1 minute to allow all traces of media to drain away. Each pellet was resuspended by swirling or gentle vortexing in 2 mL of ice-cold 0.1 M $CaCl_2$ solution for each 50 mL culture. At this point cells can be used directly for transformation or dispensed into aliquots and freezed at -70 ^{0}C. Cells were stored at 4^{0}C in $CaCl_2$ solution 24- 48 hours. The efficiency of transformation increased to four to six fold during the first 12- 24 hours of storage thereafter decreases to original level.

- Transformation

To transform the $CaCl_2$ – treated cells directly, 200µl of each suspension of competent cells was transferred to a sterile, chilled polypropylene tube using a chilled micropipette tip.

DNA (no more than 50ng in a volume of 10µl or less) was added to each tube. Contents of tube were mixed by swirling gently and stored on ice for 30 minutes. Tubes were then transferred to a rack placed in a preheated 42^0C circulating water bath for exactly 90 seconds. Tubes were rapidly transferred to an ice bath and cells were allowed to chill for 1-2 minutes. 800µl of LB medium was added to each tube and cultures were incubated for 45 minutes in a water bath set at 37^0C to allow bacteria recover and to express the antibiotic resistance marker encoded by plasmid. Appropriate volume of transformed competent cells was transferred onto agar LB medium containing 20mM $MgSO_4$ and appropriate antibiotic.

Plates were stored at room temperature until all liquid gets absorbed. Plates were inverted and incubated at 37^0C. Transformed colonies would appear in 12- 16 hours.

- Electroporation

Making electrocompetent cells:

5 mL of an overnight cultures of lactobacilli isolates SRN3 and SRN4 were diluted into fresh media MRS. The culture was grown at 30 °C for approx. 3-4 hours with shaking until reaching an $O.D._{600}$ of 0.85. The cells were pelleted by centrifugation (4 °C) and washed twice with 10 mL chilled $MgCl_2$ (10 mM) and once with 10 mL of a chilled solution of sucrose (0.5 M) and glycerol (10 % w/v). Cells were resuspended in 2-3 mL of the same solution and store until use in an ice bath, but no longer than 4 h.

Electroporation of cells:

100 ng (5µl) of pRV85 was diluted in 200µl of freshly prepared competent cells. The mixture was homogenized by gently mixing with pipette several times. The mixture was transferred into a pre-chilled cuvette. The moisture was wiped from cuvette and the cuvette was inserted into the device.

Electroporation:

Voltage (V): 1,300 V

Time constant (t): 5 ms

1 mL of pre-warmed MRS medium was immediately added and incubated at 30 ºC for 3 hours with shaking. The culture was plated on selective MRS agar plates and incubated at 30 ºC for 48 h.

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