IDENTIFICATION OF ANTI-PATHOGENIC FACTORS OF FOOD DERIVED *LACTOBACILLUS* SPECIES

BY: -

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CERTIFICATE

This is to certify that the work titled **"Identification of anti-pathogenic factors of food derived *Lactobacillus* species"** submitted by **"Ms Pranita Atri(101709) and Ms Kanika Jauhari(101724)"** in the partial fulfilment for the award of degree of Bachelor of Technology (Biotechnology) of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institution for the award of this or any other degree or diploma.

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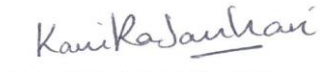
SUMMARY

Ever since Louis Pasteur discovered Lactic Acid Bacteria; they have been a great source of scientific discussion and manipulation. However, their role as effectors of anti-pathogenic effects has not yet been deciphered fully. The study presented here aimed at finding out identifying these factors and evaluating their effects against various pathogenic strains with the help of four food derived lactic acid bacteria- *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus fermentum*. Through the review of literature it was found out that the main factors responsible for inducing the anti-microbial effects are Lactic Acid and anti-microbial peptides like Bacteriocins. Through this study the quantification and the effects of these factors against four pathogenic strains (*E.coli*, *S. typhii* and two strains isolated from patients suffering from entero-pathogenic disorders were taken and represented here as B4iH and 76iH). The results obtained showed that *Lactobacillus delbrueckii*, which produced the maximum percentage of lactic acid at 72 hours, showed the maximum zone of inhibition against the entero-pathogens. Among the four strains, *Lactobacillus rhamnosus* has the best activity with respect to bacteriocin. With these results we can also conclude that the most likely pathogen against which the Lactobacillus species act, is *E.coli*. We could also see that out of the two factors the bacteriocins showed better results. Further, we also used the study to solve the problem of non-availability of structures of bacteriocins. The structures were modelled using MOE software and homology modelling techniques. The errat percentage signifies how good a modelled structure is. Since, this percentage for Sakacin G modelled structure is 100% we can conclude that this is the best possible modelled structure. The model generated were further analyzed by bioinformatics tool, Conserved Domain Search, and were found to have the same structural domain (PFAM 01721) confirming they all belong to the class(IIA) bacteriocin.

PRANITA ATRI



KANIKA JAUHARI

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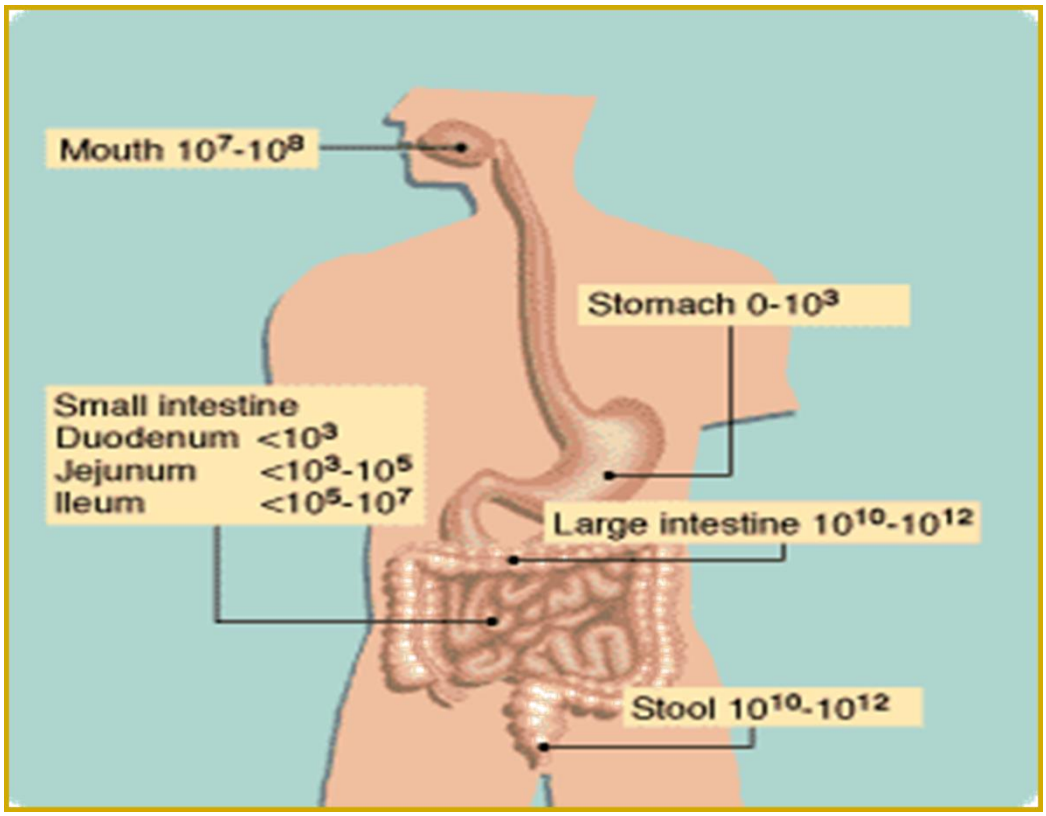
1. INTRODUCTION

The human gastrointestinal tract gives shelter to a complex and diverse ecosystem of micro biota or commensal micro flora. The microbial population ranges from 10^{12} to 10^{14} CFU/g of the human content. They play an important function in human health and disease. Gastrointestinal infections by food borne pathogens are a main cause of morbidity and mortality worldwide. Probiotics have emerged as alternative bio-therapeutic agents against intestinal pathogenic infections.

FIG 1: **The microbial count of the alimentary canal (R Fuller, 1992)**



Probiotics, as defined in a FAO/WHO (2002) report, are live 'microorganisms which when administered in adequate amounts confer a health benefit on the host'.

Probiotics are beneficial bacteria as they favourably alter the intestinal micro-flora balance such as reconstruction of normal intestinal micro-flora after it is disturbed by varied reasons like diarrhoea, antibiotic therapy (safety concerns of anti-biotic) and radiotherapy. Various workers have also reported their activity against harmful bacteria, their enhancing effect towards the immunity of the host and increased resistance to infection hence an increased immunity (Patricia *et al*, 2002, Helland *et al*, 2004). Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immune-stimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients (Grajek *et al*, 2005; Parvez *et al*, 2006).

The two most well studies genera of probiotics: *Lactobacillus* and *Bifidobacterium* are known to provide protection to the host against pathogenic bacteria. The reason for this kind of anti-microbial action is production of anti-microbial substances (mainly bacteriocins), competitive exclusion (competition for nutrients) and enhancement of the immune response of the host (W.A. Walker, 2008).

Enteric diseases (the diseases of the gastro-intestinal tract) are caused by several pathogens like few members of the *Salmonella* species, *Escherichia coli*, *Shigella*, *Listeria monocytogenes* and *Vibrio cholerae*. The declined birth rates and longer life expectancy in developed countries have led to increased prevalence of chronic disorders like cardiovascular disease and different metabolic disorders (WHO, 2003). All this requires population based new preventive approaches, namely infection control and improved

nutrition. Functional food is the food that contains some health-promoting components beyond traditional nutrients.

This study made our research group to identify the anti-pathogenic properties of the novel probiotics strains against *Salmonella typhii* and *Escherichia coli.* Also, two strains isolated from diarrhoea patients were also used for the study.

2. AIM OF THE STUDY

The aims of our present study are:

1. Estimation of Lactic Acid in four strains of Lactic Acid Bacteria: *Lactobacillus delbrueckii, Lactobacillus fermentum , Lactobacillus plantarum* and *Lactobacillus rhamnousous*

1.1Evaluation of Anti-pathogenic effects of Lactic Acid on *E. coli* and *Salmonella typhi*

2. Estimation of bacteriocins in the same 4 strains.

2.1Evaluation of Anti-pathogenic effects of bacteriocins with *E. coli* (2 strains),*Salmonella typhi* and one yet to be characterized strain isolated from patients suffering from diarrhoea.

3. Through the use of bioinformatics tools the study of the applicability of bacteriocins as food additives for preservation purposes.

3. REVIEW OF LITERATURE

3.1 FERMENTED FOODS

The history behind fermented foods:

Roman people consumed “sauerkraut” because of its taste and benefits to their overall health. Ancient Indians and modern too enjoy a before-dinner yogurt drink called as “lassi.” Bulgarians are known both for their health and their high consumption of fermented milk and kefir. In Asian cultures, pickled fermentations of cucumbers, onions, squash, and carrots, cabbage, turnips, eggplant, still exist today. People of the Ukraine consume probiotics from foods like sauerkraut, raw yogurt, and buttermilk.

These dietary habits existed from before but these fermented food products got a kick start as commercial probiotics only in the 1950s.

Fermented foods are those that have been subjected to the action of micro-organisms or enzymes, in order to bring about a desirable change. Fermented food usually provide many health benefits which are attributed to their health benefits.

Micro-organisms cause changes in the foods which:

- Help to preserve the food
- Extend shelf-life considerably over that of the raw materials from which they are made
- Improve aroma and flavour characteristics
- Increase its vitamin content or its digestibility compared to the raw materials

3.2 PROBIOTICS

Probiotic is derived from the Greek word meaning "supporting or favouring life." Lilly and Stillwell first described probiotics as "selective non-pathogenic living microorganisms, including some commensal bacterial flora, which have beneficial effects on host health and disease prevention and/or treatment". In 1900s, the Russian scientist Elie Metchnikoff stated that the Balkan population enjoyed excellent health due to consumption of fermented milks containing beneficial bacteria. These "beneficial bacteria" were later defined as "Probiotics".

Major pre-requisite properties for a microbe to be accepted as a probiotics are:

- It should be non-pathogenic, non-toxic and non-allergic.
- It should be capable of surviving and metabolizing in upper G.I. tract secretion in the gut environment e.g. Resistant to low pH, organic acids, bile juice, saliva and gastric acid.
- It should be human in origin, genetically stable and capable of remaining viable for long periods in field condition.
- It should be able to modulate immune response and provide resistance to disease through improved immunity or by the production of antimicrobial substance in the guts.
- It should have a good adhesion/ colonization to human intestinal tract and influence on gut mucosal permeability.
- It should be antagonistic against carcinogenic/ pathogenic organism.
- It should posses clinically proven health benefit, e.g. gastrointestinal disorders, persistent diarrhoea, clostridium difficle colitis, antibiotics associated diarrhoea, acute infantile gastroenteritis.

- It should have technologic properties for commercial viability such as stability of desired characteristics during processing, storage and transportation.

Established effects of probiotics (Roberfroid, 2000)

- Aid in lactose digestion
- Resistance to enteric pathogens
- Anti-colon cancer effect
- Anti-hypertensive effect
- Small bowel bacterial overgrowth
- Immune system modulation
- Blood lipids, Heart disease
- Urogenital infections
- Hepatic encephalopathy

Effects of probiotics on pathogenic bacteria (Sanders, 2003)

- Probiotics reduce plasma levels of bacterial endotoxin concentrations, by inhibiting translocation of bacteria across the GI lumen into the bloodstream.
- Decreases in translocation of bacteria may occur as a result of the ability of probiotics to tighten the mucosal barrier.
- Probiotics disallow colonization by disease-provoking bacteria through competition for nutrients, immune system up-regulation, production of antitoxins, and up-regulation of intestinal mucin genes.
- Probiotics lower colon luminal pH and foster growth of non-pathogenic commensal bacteria by SCFA (Short Chain Fatty Acid) production
- Probiotics exert protective effects through production of hydrogen peroxide and benzoic acid, which inhibit many pathogenic, acid-sensitive bacteria .

3.3. BENEFICIAL INTESTINAL BACTERIA

The intestines contain an ecosystem composed of the intestinal mucosa, the digestive secretions and the commensal micro-biota. The normal gut ecosystem can efficiently block intrusion of many pathogenic bacteria. This has been termed ‘microbial interference’ or ‘colonization resistance’

Lactobacillus sp. and *Bifidobacterium* sp. are microorganisms that form part of the human micro-biota, having an important role in the first line of defence against opportunistic and

invasive pathogens (Stecher and Hardt, 2008). Moreover, the diseases and disorders such as inflammatory bowel disease, irritable bowel syndrome and obesity are associated with human gut microbiota where aberrations could be improved by consuming probiotic lactobacilli and bifidobacteria (Fujimura *et al.*, 2010). The underlying mechanisms depend on particular functional properties of different strains of the mentioned genera and species.

3.3.1. *Lactobacillus* species

In earlier days the term LACTIC ACID BACTERIA (LABs) was used to describe milk souring organisms. Similarities between milk-souring organisms and other lactic acid bacteria were soon observed. The monograph by Orla-Jensen (1919) is said to be the base of the present classification of LABs. The principles used by Orla-Jensen were cellular morphology, mode of glucose fermentation, temperature ranges for optimized growth, and sugar utilization patterns. A number of LAB genera were recognized by Orla-Jensen including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*.

LABs have traditionally been used as food and feed starter cultures and are generally considered highly beneficial micro-organisms, some strains are even health-promoting (probiotic) bacteria. However, some genera including *Streptococcus*, *Lactococcus*, *Enterococcus*, *Carnobacterium* contain species or strains that have been proved as human or animal pathogens. Common Genera of LABs and Their Differential Characteristics are given in the table (Atte Von Wright and Lars Axelsson).

TABLE 1: Common Genera of LAB and Their Differential Characteristics are given in table(Atte Von Wright and Lars Axelsson)

Family	Genera	Characteristics								
		Shape	CO_2 from Glucose	Growth at 10°C	Growth at 45°C	Growth in 6.5% NaCl	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 9.6	Type of Lactic Acid
Aerococcaceae	*Aerococcus*	Cocci (tetrads)	–	+	–	+	–	–	+	L
Carnobacteriaceae	*Carnobacterium*	Rods	–	+	–	ND	–	ND	–	L
Enterococcaceae	*Enterococcus*	Cocci	–	+	+	+	–	+	+	L
	Tetrageonococcus	Cocci (tetrads)		+	–	+	+	–Variable	+	
	Vagococcus	Cocci		+	–	–	–		–	
Lactobacillaceae	*Lactobacillus*	Rods	Variable	Variable	Variable	Variable	–	Variable	–	D, L, DL
	Pediococcus	Cocci (tetrads)	–	Variable	Variable	Variable	–	+	–	L, DL
Leuconostocaecae	*Leuconostoc*	Cocci[a]	+	+	–	Variable	–	Variable	–	D
	Oenococcus		+	+	–	Variable	–	Variable	–	D
	Weissella		+	+	–	Variable	–	Variable	–	D, DL
Streptococcaceae	*Lactococcus*[b]	Cocci	–	+	–	–	–	Variable	–	L
	Streptococcus		–	–	Variable	–	–	–	–	L

Note: ND, not determined.

[a] Some *Weissella* strains are rod shaped.
[b] In older literature lactococci are referred to as Group N streptococci.

LABs constitute a group of gram-positive bacteria consolidated by certain metabolic, morphological and physiological characteristics. They are non-sporulating, non-respiring but aero-tolerant cocci or rods, which produce lactic acid as one of the main fermentation products of carbohydrates. They lack genuine catalase and are devoid of functional heme-linked electron transport systems or cytochromes. According to the current taxonomic classification, they belong to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales.* (Kandler and Weiss, 1986; Klein *et al.*, 1998; Holzapfel *et al.*, 2001; Axelsson, 2004).

LAB do not possess a functional respiratory system, hence they have to obtain their energy by substrate-level phosphorylation. They ferment hexoses by two basic fermentative pathways.
1. The homo-fermentative pathway takes its ground from glycolysis (or Embden–Meyerhof–Parnas pathway) and produces only lactic acid.
2. Hetero-fermentative or hetero-lactic fermentation (also known as pentose phosphoketolase pathway, hexose monophosphate shunt, or 6-phosphogluconate pathway) produces Carbon dioxide and ethanol or acetate along with lactic acid.
Lactobacillus can be classified on the basis of the mode of sugar fermentation(Hutkins et al)

- Group I, the obligatory homo-fermentative species, ferment sugars through glycolysis to yield lactic acid as the end product. Species that belong to this group can switch from homo-fermentative fermentation to hetero-fermentative fermentation under some circumstances.

- Group II lactobacilli include the facultative hetero-fermentative species, which ferment sugars to yield lactic acid as the major end product plus ethanol and CO2 in equimolar amounts if no other electron acceptor is available.
- Group III species are obligatory hetero-fermentative species, which can use both glycolysis and the pentose phosphate pathway.

TABLE 2: Classification of lactic acid bacteria

Homofermentative	*Facultative Heterofermentative*	*Obligatory Heterofermentative*
L. acidophilus	*L. casei*	*L. brevis*
L. delbrueckii	*L. curvatus*	*L. bunchneri*
L. helveticas	*L. plantarum*	*L. fermentum*
L. salivarius	*L. sakei*	*L. reuteri*
		L. pontis

3.3.2. *Lactobacillus* in food and feed

Lactobacilli have been an integral part of the human diet since its inception. Earlier, due to lack of means of preservation, the stored food became naturally fermented. Traditional fermented foods include Korean Kimchi and Caucasian kefir. Today, they are used as starter cultures in food fermentation like fermented dairy products-yoghurt, cheese, fermented milk. In alcoholic drinks like beer and wine, they provide flavour. *L. sakei* is used in fermented meat products.

Lactobacilli are also used as commercial probiotics-health enhancing microbes. Probiotics may be fermented food like yoghurt-strains are *Lactobacillus delbrueikki* subspecies *bulgarius* and *Streptococcus thermophiles* and commercially available non-fermented food like probiotic drink-YAKULT-strain is *Lactobacillus casei* strain *shirota.* Fermentation is also useful in animal feeds. *L. plantarum* and *L. buchneri* are used in silage, a fermented animal feed (Hu *et al*.2009)

3.4. GASTROINTESTINAL PATHOGENS

Enteric pathogenic infections are a main cause of morbidity and mortality worldwide. It has been recorded that severe diarrhoea and dehydration caused the deaths of 1,575,000 children under the age of five in 2006—15% of the 10.5 million deaths per year of children in this age group. Probiotics have been applied as alternative and bio-therapeutic agents for prevention of and therapy for gastrointestinal pathogenic infections.

3.4.1 *Escherichia coli*

Escherichia coli is a frequent cause of life-threatening bloodstream infections and other common infections, such as urinary tract infections. Antibiotic resistance rates in *E. coli* are rapidly rising, especially with regard to fluoro-quinolones and third- and fourth-generation cephalosporins. Surprisingly, most of these multidrug-resistant strains are acquired in the community rather than in healthcare settings. (Peter Collignon *et al*)

Drug-resistant *E. coli* are readily acquired via the diet (food and water), and there is a major turnover of drug-resistant *E. coli* each day. When people eat sterile food, there is a rapid and substantial fall in the numbers of drug-resistant *E. coli* these people carry.

This is what led us to believe to think that probiotics working against *E.coli* would be a big help. Hence, the study began.

3.4.2 *Salmonella typhii*

Salmonella is a major food-borne pathogen normally found in many food products. It causes many human diseases such as gastroenteritis, enteric fever, bacteremia, focal infections and enterocolitis. Human salmonellosis has become an important international public health and economic issue. Continual use of antimicrobial agents for treatment of salmonellosis may result in the emergence of antibiotic-resistant strains of Salmonella. This multi-drug resistance has caused great public health concern.

The study by Thirabunyanon *et al*. showed that lactic acid bacteria isolated dairy products suppress the growth of *Salmonella typhimurium* and *Salmonella enteritidis*.

3.5 FUNCTIONAL FOOD

Diet and nutrition are important factors in the promotion and maintenance of good health throughout the entire life-course. However, rapid changes in diets and lifestyles have a significant impact on the health and nutritional status of populations. While the standards of living have improved, food availability has expanded and become more diversified. There have also been significant negative consequences in terms of inappropriate dietary patterns, decreased physical activities and increased tobacco use, and a corresponding increase in non communicable diet-related chronic diseases (*e.g*. obesity, diabetes mellitus type 2, cardiovascular disease, hypertension and stroke, and some types of cancer) (WHO, 2003).

Functional food (FF) is a natural food, to which a component has been added/removed or a food in which the bioavailability of the components has been modified by technological or biotechnological means (Roberfroid, 2000). FF includes conventional foods, modified foods (fortified, enriched, or enhanced), medical foods, and foods for special dietary use (Siro *et al.*, 2008; Hasler and Brown, 2009). FF can play an important role in the risk reduction of non-communicable diseases and can prolong remission in IBD (including Crohn's disease and ulcerative colitis) and alleviate allergic conditions by providing benefits beyond usual nutrition as well as in optimising health and general well-being (ILSI, 2009; Fujimura *et al.*, 2010).

Fortification of food with these probiotic strains would be a big help.

3.6 MECHANISM OF ACTION OF PROBIOTICS

Production of Antimicrobial substances

Production of antimicrobial compounds is very helpful as these act as direct antagonists against entero-pathogens. Bacteriocins are ribosomally synthesized antimicrobial peptides. Pediocin, said to be a representative of Class IIA bacteriocins, secreted by *Pediococcus acidilacti* MM33 was bactericidal against *Listeria monocytogenes* (Millete *et al*). Other inhibitory factors of *Pediococcus* spp are hydrogen peroxide, lactic acid, exopolysaccride, photolytic activity(Z. Yuksekdaget *et al*,2010). Production of short chain fatty acid (SCFA) such as acetic and lactic acid lowers the pH leading to inhibition of growth. A bio-surfactant produced by *Lactobacillus paracasei* exhibits antimicrobial activity.

Competition for nutritional substrates

Probiotic population in the GI tract increases when humans consume nutrients. Therefore, competition between probiotics, intestinal pathogens and microbes occur. *Bifidobacterium* inhibited the growth of *Porphyromonas* by competing for vitamin K growth factor.

Competitive Exclusion

Probiotics by the process of competitive exclusion eliminate entero-pathogens at the adhesion and infection site of the epithelial cells in the human intestine by competing for the glycol-conjugate receptors. The initiation of infection takes place with the binding of the entero-pathogen to the intestinal epithelium through interaction between bacterial lectins and carbohydrate moieties of glycol-conjugate receptor molecules on the intestinal epithelium. *L. plantarum* prevents the adhesion of Clostridium sporo-genes by competitive exclusion (Ramiah *et al*,2008).

Enhancement of intestinal barrier function

Epithelial junction complexes consist of tight junctions which function as permeability barrier as well as a fence to maintain difference between apical and basolateral domains in the plasma membrane. Breakdown of tight junction allows the penetration of materials from the lumen to adlumenal compartment of epithelium. During infection, the entero-pathogens attach to luminal surfaces of the host epithelium, effacing the localized regions of microvilli, leading to infection of the bacterial effector proteins via a syringe like type III secretion system. The function of effectors proteins is to anchor the bacteria to host cells causing inflammation (J.A Guttman). Probiotics promote the increase in the intestinal barrier function by extirpating the translocation and attachment of pathogens to the intestinal epithelium (C.Reiff *et al*).

The intestinal epithelium modulates the intestinal environment by producing cryo-protective substances. Heat shock proteins are constitutively expressed in the epithelium, however their level increases under stress to maintain intestinal homeostasis and defence against injury. *B.thetaiotamicron* trigger the Paneth cells to release angiogenin 4(Ang 4) which shows bactericidal activity against pathogens. Apotosis is an important factor in the colonic inflammatory diseases. Probiotics prevent the cytokine-induced epithelial damage by promoting intestinal epithelium cell survival.

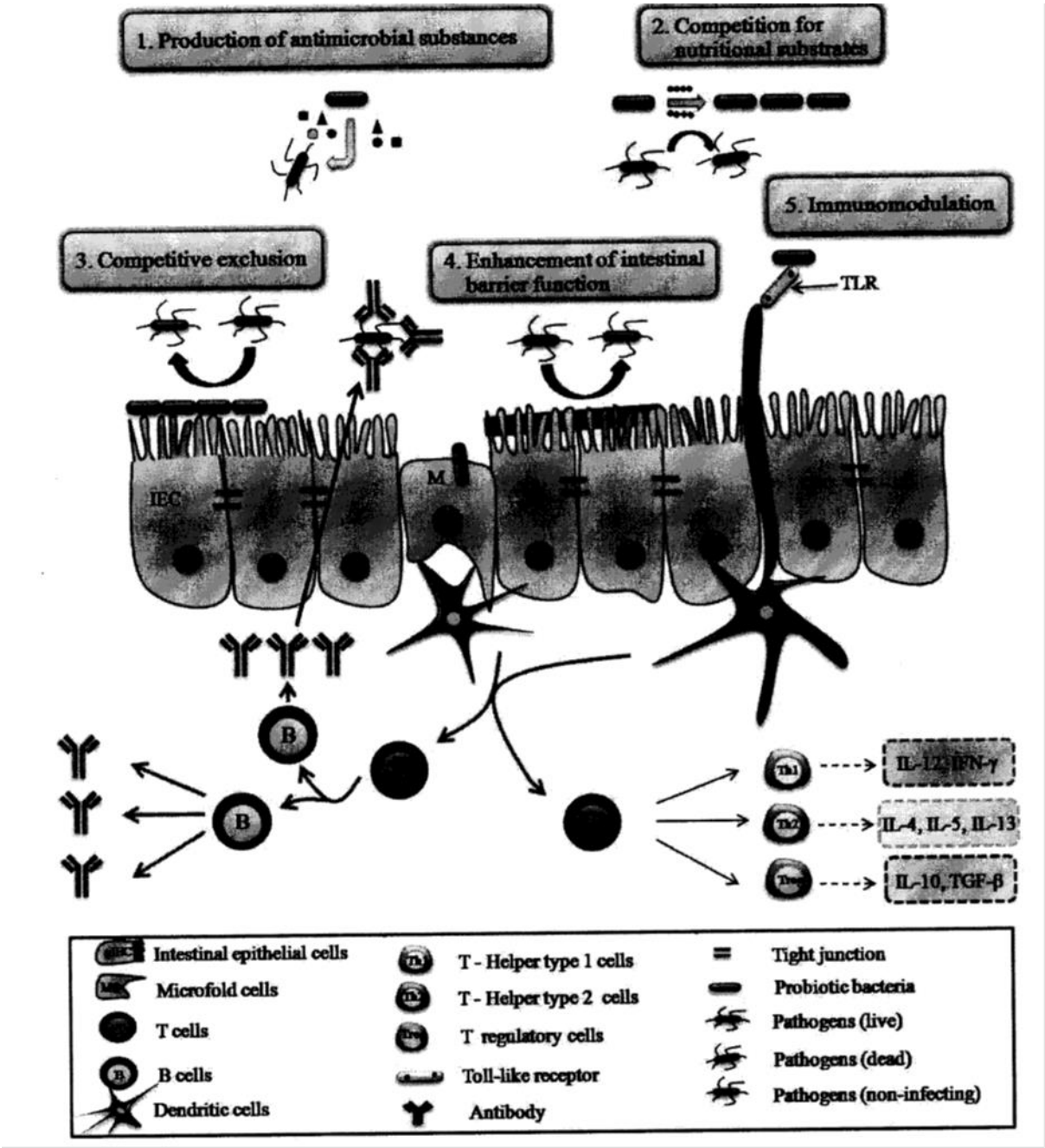
Immunomodulation

Innate immunity prevents invasion of pathogenic and commensal microbes. Probiotics have the potential to promote innate immune response against dietary antigens and microbes. Dendritic cells of the intestine retain commensal bacteria by exclusively activating B lymphocytes to produce IgA to decrease the mucosal penetration by bacteria. Commensals carried by the dendritic cells are restricted to the intestinal mucosal lymphoid tissue, avoiding possible systemic immune response.

The host innate defence system has to regulate the responses according to the level of threat of any pathogen. A weak response causes the non-clearance of an infection, increasing the possibility of a systemic infection. A strong response may cause surplus tissue damage. Dendritic cell maturation increases the secretion of cytokines and expression of molecules required for the activation of T and B cells. Probiotics stimulate the dendritic cells to produce anti inflammatory cytokines-IL-10 to reduce Th1 response. Probiotic mixture VSL#3 produces IL-10 in human and murine dendritic cells.

Probiotics inhibit pro-inflammatory cytokines. *L.rhamnosus* induces high levels of granulocyte colony stimulating factor production from macrophages which is required for the suppression of *E.coli*, *L.johnsonii*, *L.gasseri*, *L.reuteri* and increase IL-12, IL-18 production by dendritic cells. These dendritic cells which are exposed to *Lactobacillus* alter CD+4 and CD+8 T cells to Th1 and Tc1 polarization to increase IFN-y production. In this way the commensal microbes and probiotic strike a balance between pro- and anti-inflammatory mucosal responses maintaining intestinal homeostasis (Mohamedzadeh M *et al*).

FIG 2: Mechanism of action of probiotics



3.6.1 ANTIMICROBIAL COMPONENTS OF LACTIC ACID BACTERIA

- Organic Acids-Lactic Acid
- Bacteriocins

3.6.1.1 ORGANIC ACIDS

Human GI tract consists of both aerobic and anaerobic bacteria. Obligate aerobes grow in presence of oxygen producing ATP. Facultative Anaerobes undergo either aerobic respiration or fermentation. Obligate aerobes grow in absence of oxygen and undergo fermentation producing Short Chain Fatty Acids(SCFAs). These bacteria use carbohydrates as the main source of energy. Lactic Acid is one of the most important SCFAs produced as it exhibits a high amount of antimicrobial activity. The mode of action of lactic acid includes one of the following :

The production of lactic acid causes a drop in the pH of the fermentate, gradually causing a the breakdown of organic acids (small fatty acids(SFAs)). The un-dissociated SCFAs eventually penetrate the bacterial membranes, destroying their cytoplasm or inhibiting growth (ameliorating the bacterial decarboxylases and catalases. Intestinal dissociation liberates H+ ions which serve as pH barrier against the colonization of pathogens. The gastric Ph is reduced as compared to the inclusive HCl. The gastric hydrolysis releases H+ ions mobilizing pepsinogen and stagnating bacterial growth. (Zdzislaw Mroz)

The pH drops to 4.0 which is enough to inhibit the growth of most common pathogens thus causing increased shelf life. This acidity also makes changes in the texture of food due to the precipitation of few proteins and biochemical conversions involved in growth hence improving the flavour. These reasons favour the use of lactic acid bacteria in the food industry.

3.6.1.2 BACTERIOCINS

Bacteriocins are proteins or protein complexes synthesised by various bacterial strains. They exhibit bacteriocidal and bacteriostatic activities usually against closely related species. Bacteriocins are classified into various classes as tabulated in Table 1. Among these the most well studied one are the Class 2A which finds applications mainly in food preservation due to its efficient action against spoilage organism *Listeria monocytogenes.* It is also well known that, the lactic acid starter cultures of fermented foods display numerous antimicrobial activities which is mainly because of their ability to secrete a variety of bacteriocins. Another significant point of interest are their antimicrobial activities against pathogenic microorganisms which have been reported by several workers(Lewus *et al*,1991, Jones *et al*,2008, Ryan *et al*,1998) .Another point of importance is that the bacteriocins derived from Lactic Acid Bacteria are generally regarded as safe (GRAS) since these are food derived bacteriocins hence this feature.

TABLE 3: **Classification of Bacteriocins**

CLASS I	CLASS II	CLASS III
Lantibiotics	Non modified heat stable peptides	Protein Bacteriocin
Type A: Elongated Shaped Molecule	2a: Pediocions like Bacteriocins	IIIA:Lysis causing
Type B:Globular Molecular	2b: Two peptide bacteriocins	IIIB

The various bacteriocins which have been characterized and very well studied are given in table 4. This kind of study is important to study the applicability of the bacteriocins since knowing if they are active against gram positive or gram negative organisms is very important in order to apply these bacteriocins against various gram negative pathogenic species since most of the pathogenic strains belong to the gram negative category.

TABLE 4: **Various bacteriocins with their targets**

S.No	Bacteriocin	Gram positive	Gram negative
1	Planataricin C19	+	-
2	Leucocin-A (Leucocin A-UAL187)	+	-
3	Leucocin-B	+	-
4	Lactococcin MMFII	+	-
5	Mesentericin Y105	+	-
6	Bavaricin-A	+	-
7	Curvacin-A	+	-
8	Sakacin-A	+	-
9		+	-

	Bavaricin-MN		
10	Carnobacteriocin BM1	+	-
11	Divergicin M35	+	-
12	Leucocin C	+	-
13	Mundticin	+	-
14	Bacteriocin ST15	+	+
15	Pisciocin V1b	+	-
16	Divercin V14	+	-
17	Sakacin P	+	-
18	Enterocin P(classified in A and C)	+	-
19	Piscicolin 126	+	-
20	Divercin V41	+	-
21	Sakacin-P (Sakacin 674)	+	-
22	Enterocin P	+	-
23	Piscicolin 126	+	-
24	Pisciocin V1a	+	-
25	Pediocin PA-1 (Pediocin ACH)	+	-
26	Enterocin A	+	-
27	Carnobacteriocin B2 (Carnocin CP52)	+	-
28	Enterocin A	+	-
29	Plantaricin 423	Unknown	
30	Enterocin CRL35 (Mundticin KS)	Unknown	

31	Acidocin A	+	-
32	Listeriocin 743A	Unknown	
33	Enterocin SE-K4		
34	Penocin A	+	-
35	Ubericin A	+	-
36	Bacteriocin T8	+	-
37	Enterocin-HF	+	-
38	Avicin A	+	-
39	Mundticin L	+	-
40	Weissellin A	+	-
41	Sakacin G	Unknown	
42	Bacillocin 602	-	+
43	Bacillocin 1580	-	+
44	Bacteriocin L-1077	-	+

TABLE 5: **Mode of action**

CLASS I	CLASS II	CLAS S III	
Lantibiotics	Non modified heat stable peptides	Protein Bacteriocin	
Kill by disrupting the	Kill by sensitizing	IIIA That kill bacterial cells	IIIB Killing the target cells by disrupting the membrane potential,

integrity of the membrane.	the cell membranes	by cell-wall degradation, thus causing cell lysis	which causes ATP efflux .

The addition of bacteriocins into food matrices is dependent on their interaction with the various food components. These food components mainly peptides and fats are known to reduce the activity of these bacteriocins. Hence, choosing bacteriocins which have minimum interactions with these food matrices is of utmost importance. For such studies the laboratory experimentation available are very cumbersome and time consuming. Here the bioinformatics based approaches like protein docking studies come in handy.

For applying the various studies like the protein docking techniques it is very important for the structures of these bacteriocins to be known. The conventional methods of determination of protein structures like NMR and X-Ray crystallography based approaches present structures of very high efficiency but are highly cumbersome and time consuming. Also, the instrumentation required for these methods is very expensive and not easily available. Hence comes the importance of bioinformatics based approaches for determination of protein structures also. The various methods like homology modelling and threading can be applied based on the percentage similarity of the sequences of the proteins whose structures need to be determined and the proteins with already known structures which are already reported in the databases.

3.7 HOMOLOGY MODELLING OF PROTEINS

Homology or comparative protein structure modelling is a technique used for the modelling of protein structures when the percentage similarity is more that 50%. . The prediction process consists of fold assignment, target–template alignment, model building, and model evaluation. With the increasing efficiency of the modelling software and the increasing number of structures from various protein families being determined by the conventional methods, it has become really convenient to use these tools for protein structure modelling.

3.8 PROTEIN PURIFICATION TECHNIQUES

The activity of proteins like bacteriocins depends highly on their purity level. For the achievement of a highly pure protein a multi-step process is applied, usually. These include preliminary steps like ammonium sulphate precipitation followed by dialysis, further purified by various types of chromatographies.

Ammonium Sulphate Precipitation- It is a method to purify proteins by varying their solubility. The solubility of proteins changes according to the salt concentration. Two processes take place: “Salting in” is a process in which the solubility of protein increases as

the ionic strength (salt concentration) rises. Then, a stage arrives when on addition of salt, the solubility of starts decreasing. At a sufficiently high ionic strength, almost the entire protein is precipitated from the solution, a method known as "Salting out". Ammonium sulphate is the most common used ion as it does not hinder the activity of enzyme and water soluble.

The most important step in this is to find the ammonium sulphate concentration which will precipitate the paramount proportion of undesired protein, leaving behind the desired protein in solution or the other way round.

Amount of ammonium sulphate (in grams) to add to 1 litre of a solution at 20 °C
= **533(S2-S1)/100-0.3S2**
S2 = Final % saturation (E.g. 50 %)
S1 = Initial % saturation (E.g. 0 %; the starting material being equivalent to water)

- Dialysis- Dialysis is the method by which based on the molecular weight proteins are separated out. It is carried out by placing the solution to be purified inside membrane and placing the membrane in a hypotonic solution. Here, the lower molecular weight compound remains behind inside the membrane and the higher molecular weight compound moves out into the hypotonic solution.

Through our review of literature we felt that there is a lacuna as far as the studies related to the anti-pathogenic effects of these bacterial strains are concerned. The studies which have been conducted have been conducted on the microbial strains also have not been related to these entero-pathogens. Also, since the final aim of our research group is to get a food matrix which will, as our studies suggest, be curative for these diseases.
The general goals of our research project are:

- To identify the anti-pathogenic factors of *Lactobacillus* species.
- To inhibit the growth of entero-pathogens like *Salmonella typhii* and *Escherichia coli*
- Identify which strain out of our four lactobacilli strains(*Lactobacillus delbruekii*, *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus rhamnosus*) which has the best activity.
- Incorporate the best strain into a food matrix.

4. MATERIALS AND METHODS

An overview of the material and methods used in this study are described as follows:

4.1. MATERIALS

1. Study strains

TABLE 6: Study Strains

STRAINS	MTCC NO.
Lactobacillus delbrueckii	911
Lactobacillus fermentum	1745
Lactobacillus plantarum	2941
Lactobacillus rhamnosus	1408
Salmonella typhii	98
Escherichia coli	723

2. Chemicals

- MRS Broth (HiMedia)
- MRS Agar (HiMedia)
- 5%(w/v) Calcium Hydroxide
- 0.1N Sodium Hydroxide
- Distilled Water
- Phosphate buffer

4.2 METHODS

4.2.1 Estimation of Lactic Acid

Lactic acid estimation was carried out by the method given by Barnali Ashe *et al* (2010). Though, a few minor changes were made while doing the experiment since the results obtained were not as expected. Lactobacilli were grown in MRS broth for 24 hours,48 hours ,72 hours, 96 hours and so on till the a decrease was seen in the lactic acid production. At an interval of 24 hours an estimation of lactic acid carried out. The following steps were followed-

- The fermentate was taken and centrifuged at 7000 RPM for 10 minutes so that the cell debris is precipitated out.
- The supernatant is then collected and precipitation is carried out with 5% Calcium hydroxide solution till the time the pH reaches the value of 7.

- Then, though, the protocol mentions filtering , instead of filtering we carried out centrifugation at 7000 RPM for 10 minutes again so that whole of the lactic acid is precipitated out in the pellet.
- The pellet was then resuspended in minimum amount of water and lactic acid titration was carried out.
- For lactic acid titration standard protocol was carried out which is titrating it against sodium hydroxide using Phenolphthalein indicator.
- The percentage lactic acid was calculated using :

$$\% \text{ acid} = \frac{(\text{ml of NaOH used})\,(\text{conc. NaOH})\,(0.090 \text{ this is the milli equivalent weight of lactic acid}}{\text{Weight of Sample}}$$

- The weight taken here was the wet weight which was calculated by taking the weight of the empty tarson vial and then the weight with the pellet is taken and the 2 weights are subtracted to get the weight of the pellet.
- The calculations were done and a kinetics for lactic acid was obtained as seen in the results section.

4.2.2 Disc diffusion assay

The final aim of our project is to test whether these strains are having any anti pathogenic activity or not so this we checked by the disc diffusion assay. We grew *Salmonella typhii* and *E.coli* overnight in nutrient broth. They were then grown on to MacKonkey agar plates and discs with the 72 hour grown lactic acid (since according to the kinetics that was the point where maximum lactic acid was observed) was taken and put in the middle of the plate. The zone of inhibition was observed after 72 hours hence proving that lactic acid produced by lactic acid bacteria is actually showing an anti-pathogenic activity on these strains.

4.2.3 Bacteriocin estimation*:*

The production and estimation of bacteriocins was carried out by the following method by G. Rajaram *et al*, 2010

- The lactic acid bacteria culture was allowed to grow for 48 hours at 30oC with 5% inoculum from the overnight grown culture.
- The cells were removed from the fermentate by centrifuging it at 10,000 g for 15 minutes

- The cell free supernatant was then adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin.
- The crude bacteriocin was precipitated with 80% ammonium sulphate saturation and then the pellet was resuspended in phosphate buffer 20mM for 12 hours at 4oC
- The estimation was then carried out and results as mentioned below were found out

4.2.4 Bacteriocin structure modelling

–The aim of this part is to study the structure function relationship between bacteriocins. It was carried out in the following way:

Selection of strains

Five strains and corresponding bacteriocins(*Pediococcus acidilactici*- Pediocin AcH(Biswas *et al*,1991), *Leuconostoc mesenteroides*-Mesenterocin Y105(C.Hill), *Enterococcus mundtii*- Munditicin(Yamasaki *et al*,1991) *Lactobacillus sakei*-Sakacin G(Simon *et al*,2002), *Lactobacillus plantarum*- Plantaricin 423(Finland *et al*, 2005)) were screened out on the basis of the errat accuracy of the modelled structures of their bacteriocins. Errat is an online bioinformatics tool which can be used to verify the accuracy of protein structures. Structures with errat score of 80% above were selected.

Screening of Motif

A motif is a conserved region in a protein sequence which characterizes it as belonging to a particular class. The motif based search was also carried out for these bacteriocins using SCAN-PROSITE tool. The conserved class IIA motif "YGNGVXCXXXCXV" (Finaland *et al*, 2005) was found out in all the sequences

Screening for template structure

The template structures for these five bacteriocins were found out with the help of a sequence based BLAST search and the structure with the best combination of the query coverage and percentage similarity was chosen. The template structure for each of the bacteriocins is as represented below (Fig 3).

Fig3 (a) **Fig 3(b)**

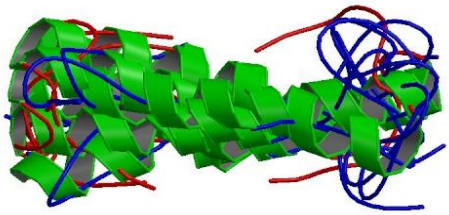


Fig 3(a): SAKACIN P (PDB ID 1OHM): Template for Pediocin Ach and Munditicin. (b): LEUCOCIN A (PDB ID 1CW6_A): Template for Mesenterocin Y105, Planatricin 423 and Sakacin G

Model Building

Based on the template structures and the sequence of the bacteriocins homology modelling was performed using MOE software.

5. RESULTS

5.1 GROWTH KINETICS:

The growth kinetics was carried out to first to be sure of the purity of the cultures obtained from MTCC and the information provided by them. Also, since in lactic acid bacteria the concentration of lactic acid produced would be in parallel to the growth that is why the growth kinetics curve will help in the further studies. Through the growth kinetics we could be sure that the lactic acid concentration should be highest at 72 hours which is in accordance to the information provided by other study groups (Bergeley *et al*). The figures below (Fig 4-7) represent the growth kinetics of the four selected strains.

FIG 4: Growth Kinetics of *Lactobacillus delbrueckii*

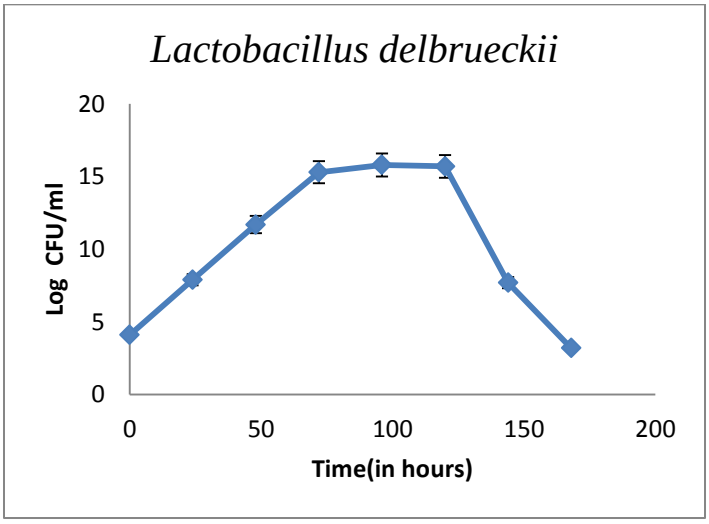


FIG 5: Growth Kinetics of *Lactobacillus plantarum*

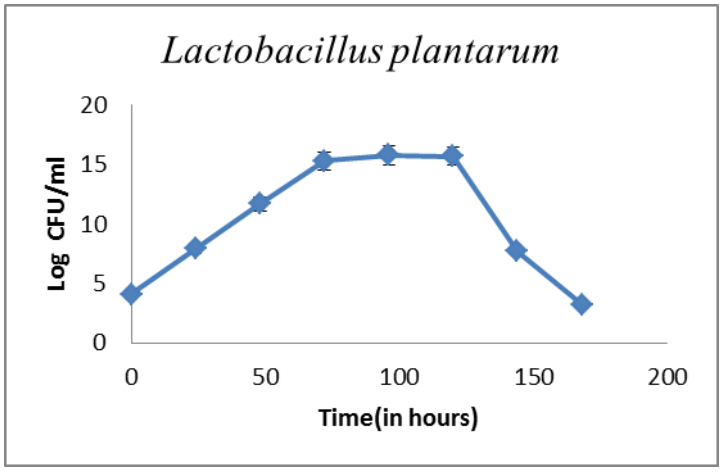


FIG 6: Growth Kinetics of *Lactobacillus rhamnosus*

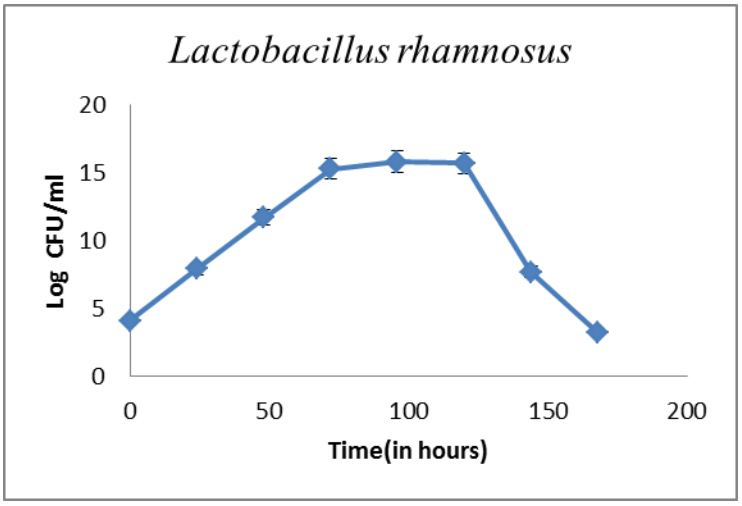
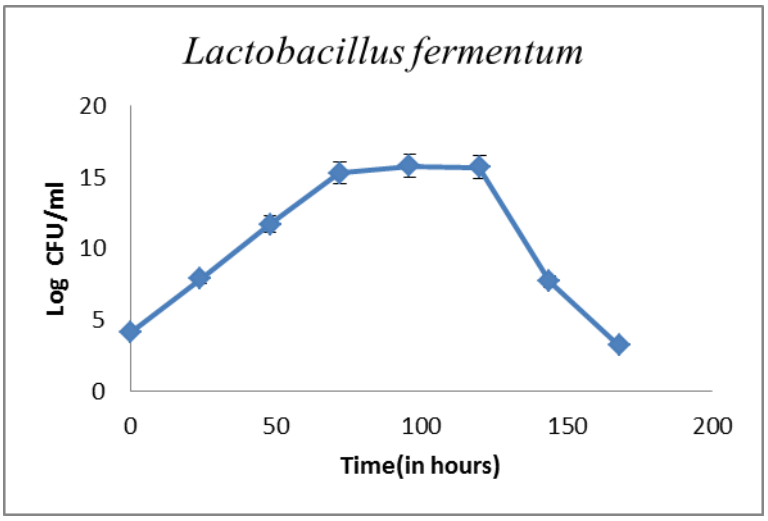


FIG 7: Growth Kinetics of *Lactobacillus fermentum*



5.2 LACTIC ACID ESTIMATION:

5.2.1 pH of the inoculum :

The pH of the medium was taken as it was since reduction in the pH signifies that lactic acid is produced in the culture. Since, the pH from 0 hour to 72 hour reduced significantly (Represented in Table 7, 8 and 9) we can say that the production of lactic acid is taking place. Also, since as mentioned above at 72 hour we expected there to be maximum growth of lactic acid this was taken as the time for the growth of culture for lactic acid estimation.

TABLE 7: Set I

Strain	pH at 0 hour	pH at 72 hour
L. delbrueckii	6.7	3.4
L.plantarum	6.6	3.6
L.rhamnosus	6.5	3.1
L.fermentum	6.7	2.7

TABLE 8: Set II

Strain	pH at 0 hour	pH at 72 hour
L. delbrueckii	6.5	3.1
L.plantarum	6.5	3.4
L.rhamnosus	6.8	3.0
L.fermentum	6.7	2.6

TABLE 9: Set III

Strain	pH at 0 hour	pH at 72 hour
L. delbrueckii	6.3	3.2
L.plantarum	6.5	3.3
L.rhamnosus	6.7	3.1
L.fermentum	6.4	3.0

5.2.2 Titre Value

The titre value is the value of NaOH used to completely neutralize the lactic acid produced in the medium. This value is need to calculate the percentage of lactic acid produced in the medium since in acid-base titrations the titre value signifies the amount of the unknown quantity out of the acid or the base. Acid base titrations are used to quantify one of the acid or the base when the details about the other are known entirely. Here, the unknown quantity is the acid value.

TABLE 10: ***Lactobacillus delbrueckii***

	24 hour	48 hour	72 hour	96 hour	120 hour
Set I	16 ml	46 ml	90 ml	110ml	65ml
Set II	20 ml	42ml	87ml	100ml	65ml
Set III	15ml	50ml	96ml	106ml	67ml

TABLE 11: ***Lactobacillus plantarum***

	24 hour	48 hour	72 hour	96 hour	120 hour
Set I	135 ml	150 ml	200 ml	100 ml	100 ml
Set II	137ml	146ml	190ml	98ml	100ml
Set III	134ml	152ml	200ml	102ml	98ml

TABLE 12: ***Lactobacillus fermentum***

	24 hour	48 hour	72 hour	96 hour	120 hour
Set I	132 ml	270 ml	400 ml	140 Ml	135 ml
Set II	130ml	265ml	388ml	142ml	140ml
Set III	135ml	268ml	398ml	148ml	136ml

TABLE 13: ***Lactobacillus rhamnosus***

	24 hour	48 hour	72 hour	96 hour	120 hour
Set I	126ml	220ml	270ml	150ml	150ml

Set II	130ml	222ml	266ml	154ml	152ml
Set III	124ml	218ml	272ml	148ml	156ml

5.2.3 Percentage of Lactic acid produced

The percentage lactic acid was determined using the titre value and the weight of the pellet. The formula used for this calculation is:

$$\% \text{ acid} = \frac{(\text{ml of NaOH used})(\text{conc. NaOH})(0.090 \text{ this is the milli equivalent weight of lactic acid}}{\text{Weight of Sample}}$$

TABLE 14: Set I

	24 hour	48 hour	72 hour	96 hour	120 hours
Lb. delbrueckii	9.42%	35.06%	66.95%	23.42%	22.68%
Lb. plantarum	13.5%	25.28%	50.70%	37.88%	20.73%
Lb. Fermentum	3.00%	8.625%	58.99%	27.88%	13.92%
Lb. Rhamnosus	18.9%	31.27%	51.20%	28.36%	21.02%

TABLE 15: Set II

	24 hour	48 hour	72 hour	96 hour	120 hours
Lb. delbrueckii	9.29%	34.31%	65.02%	23.71%	22.14%
Lb. plantarum	15.24%	24.51%	48.58%	37.69%	20.59%
Lb. Fermentum	3.75%	8.21%	56.33%	25.00%	13.70%
Lb. Rhamnosus	19.40%	31.61%	51.40%	31.64%	23.56%

TABLE 16: Set III

	24 hour	48 hour	72 hour	96 hour	120 hours
Lb. delbrueckii	9.64%	34.90%	66.70%	23.71%	22.14%
Lb. plantarum	13.37%	25.67%	50.70%	38.89%	20.36%
Lb. Fermentum	2.78%	9.76%	61.27%	26.35%	14.03%
Lb. Rhamnosus	18.60%	31.00%	51.75%	30.27%	23.56%

5.2.5 Antimicrobial activity

The anti-microbial activity was determined by the well diffusion method. The pathogenic strains were grown on MacKonkey agar plates and the 72 hour grown culture was put into wells. The results are as represented below (Table 17, Fig 8-11)**.**

TABLE 17: Antimicrobial activity of 72 hours grown culture

	Lb. delbrueckii	*Lb. plantarum*	*Lb. Fermentum*	*Lb. Rhamnosus*
E.coli	2.0cm	1.5cm	1.7cm	1.7cm
Salmonella typhii	1.7cm	1.3cm	1.3cm	1.3cm

FIG 8: Antimicrobial Activity of *Lactobacillus delbrueckii* with *E.coli* and *S.typhii*

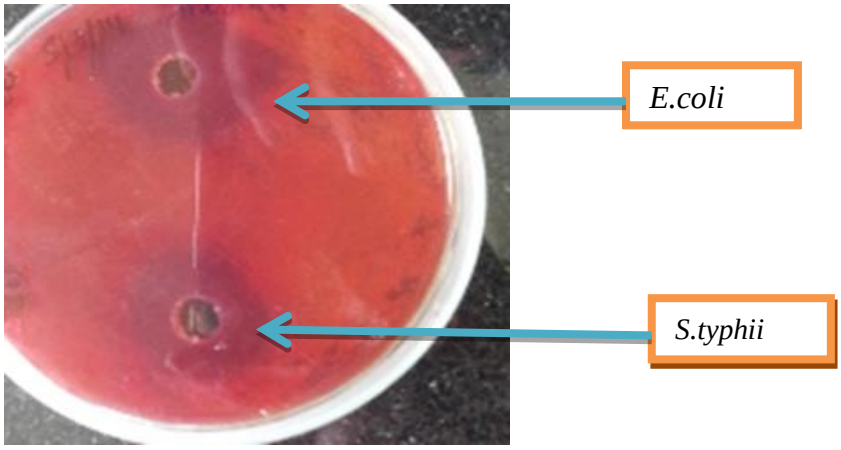


FIG 9:Antimicrobial Activity of *Lactobacillus plantarum* with *E.coli* and *S.typhii*

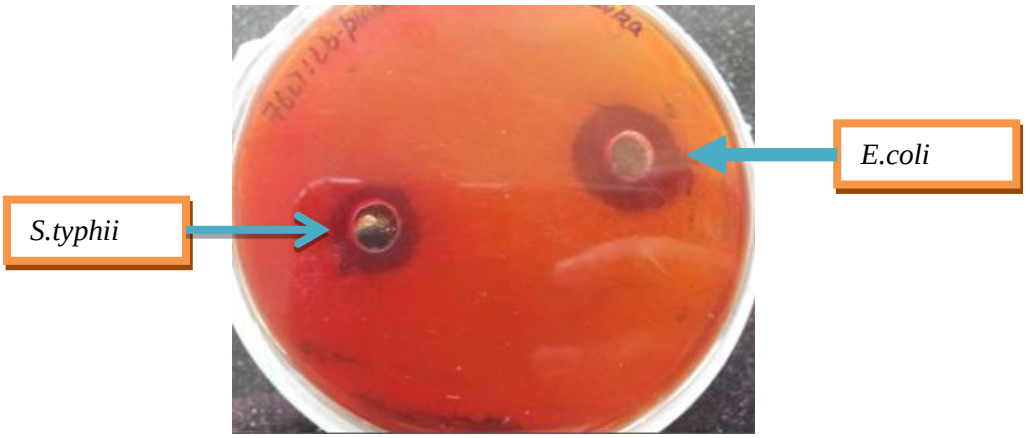


FIG 10:Antimicrobial Activity of *Lactobacillus rhamnosus* with *E.coli* and *S.typhii*

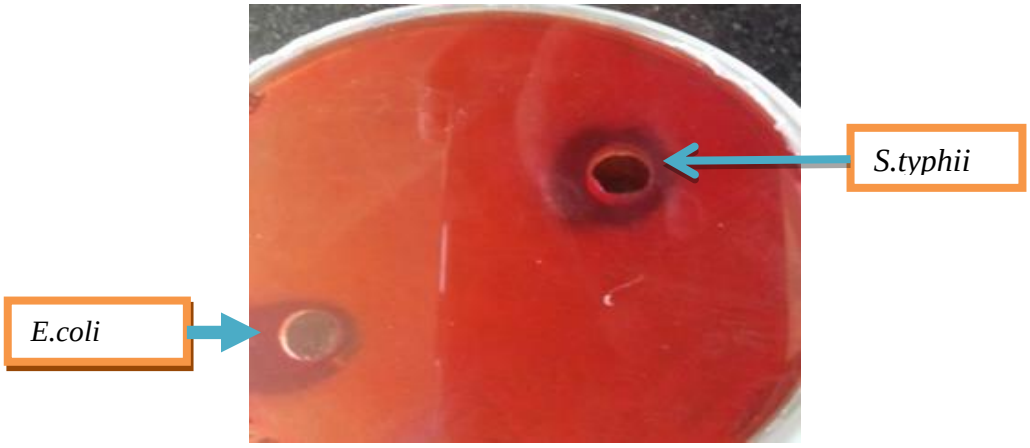
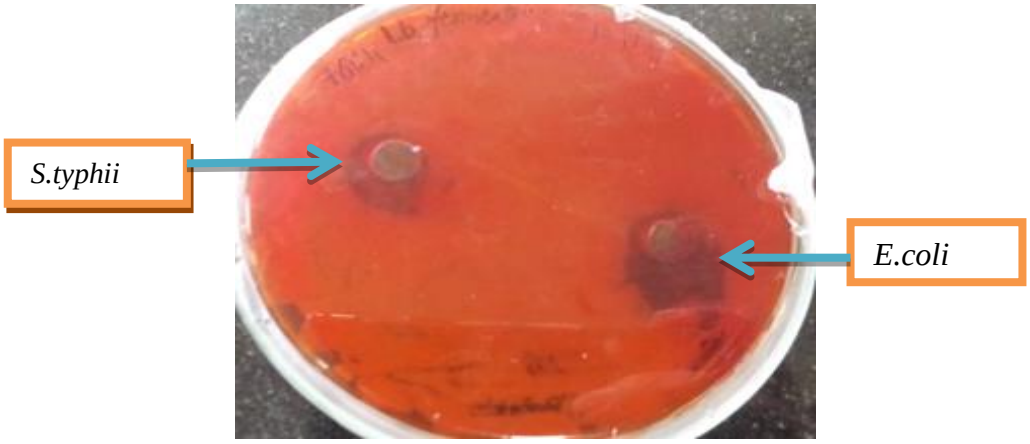


FIG 11:Antimicrobial Activity of *Lactobacillus fermentum* with *E.coli* and *S.typhii*



5.3 BACTERIOCINS

The antimicrobial spectrum of bacteriocins was determined by well diffusion assay. Three strains of *E.coli and S.typhii* were spread on the plates containing MacKonkey Agar. The purified bacteriocins were put into the wells. These were co-incubated for 24 hours and the zone of inhibition was measured. The results have been represented below (Table 18, Fig 12-27).

TABLE 18: **Zone of inhibition with bacteriocins of *Lactobacillus* species**

Strain	*E.coli*(MTCC 723)	*E.coli*(76iH)	*E.coli*(B4iH)	*S.typhii*
Lb. delbrueckii	2.1cm	0.8cm	0.8cm	1.5cm
Lb. plantarum	1.7cm	0.5cm	0.5cm	1.6cm
Lb. fermentum	2.2cm	1.2cm	1.2cm	1.8cm
Lb. rhamnosus	2.5cm	1.4cm	1.3cm	1.8cm

FIG 12: Antimicrobial Activity of *Lactobacillus delbrueckii* with *E.coli*

FIG 13: Antimicrobial activity of *Lactobacillus delbrueckii* with strain B4iH

FIG 14: Antimicrobial activity of *Lactobacillus delbrueckii* with strain 76iH

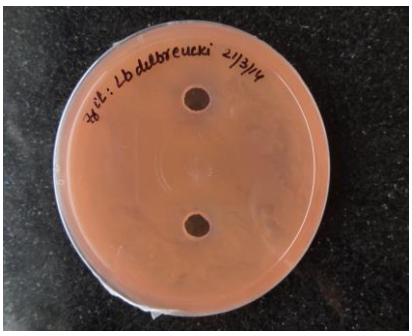


FIG 15: Antimicrobial activity of *Lactobacillus delbrueckii* with *S.typhii*

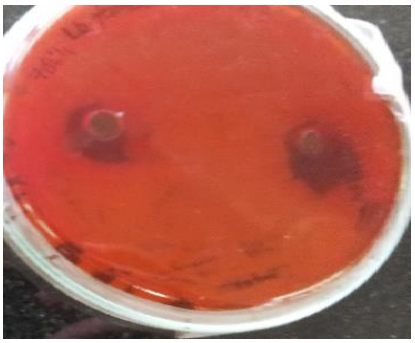


FIG 16: Antimicrobial Activity of *Lactobacillus plantarum* with *E.coli*

FIG 17: Antimicrobial activity of *Lactobacillus plantarum* with strain B4iH

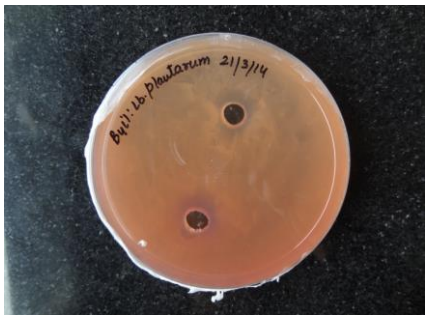


FIG 18: Antimicrobial activity of *Lactobacillus plantarum* with strain 76iH

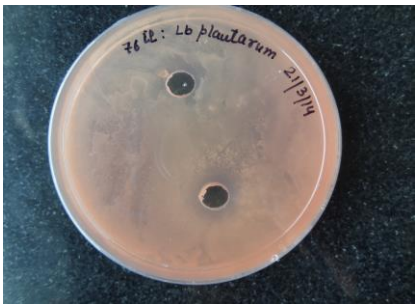


FIG 19: Antimicrobial activity of *Lactobacillus plantarum* with *S.typhii*

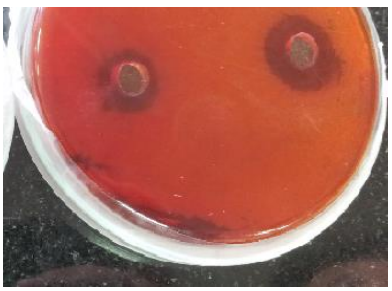


FIG 20: Antimicrobial activity of *Lactobacillus rhamnosus* with *E.coli*

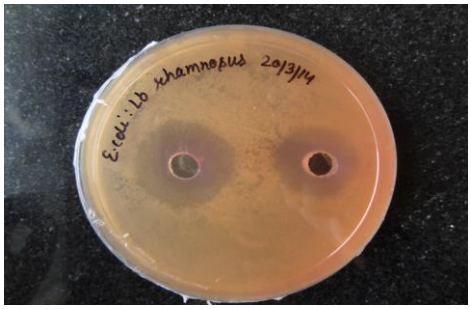


FIG 21: Antimicrobial activity of *Lactobacillus rhamnosus* with strain B4iH

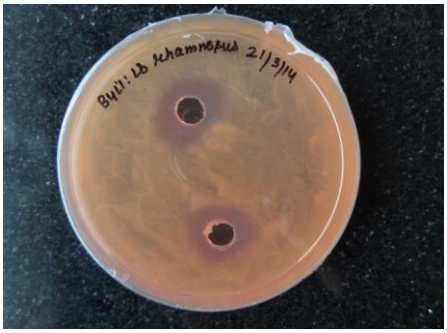


FIG 22: Antimicrobial activity of *Lactobacillus rhamnosus* with strain 76iH

FIG 23: Antimicrobial activity of *Lactobacillus rhamnosus* with *S.typhii*

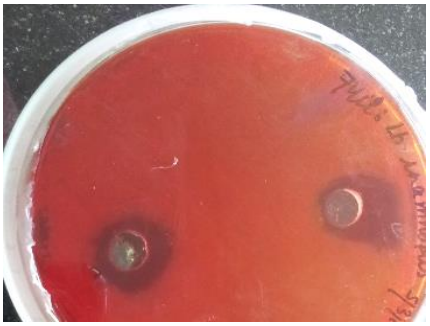


FIG 24: Antimicrobial Activity of *Lactobacillus fermentum* with *E.coli*

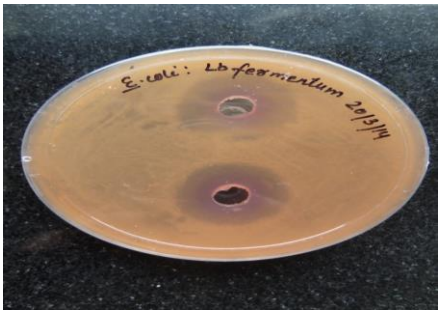


FIG 25: Antimicrobial activity of *Lactobacillus fermentum* with strain B4iH

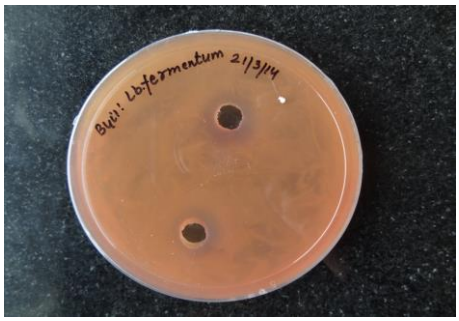
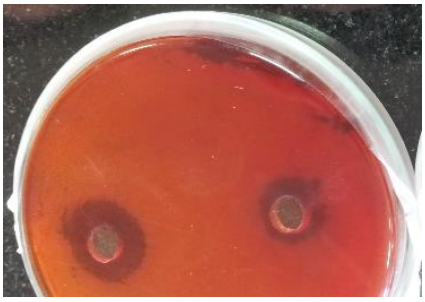


FIG 26: Antimicrobial activity of *Lactobacillus fermentum* with strain 76iH

FIG 27: Antimicrobial activity of *Lactobacillus fermentum* with *S.typhii*



5.3.1 Bacteriocin structure modelling

Bacteriocins are well known anti-microbial agents which have been used as food preservatives (Cleavaland *et al*, 2001). However, one of the limiting factors in the application of bacteriocin is their stability and efficacy in the food matrix. The protein stability studies are dependent on availability of the three dimensional structures given by NMR and X-RAY crystallography techniques which may not always be avalaible. In silico based approaches provide a convenient method for the in depth studies and prediction of their functionality.

In this study we present the three dimensional homology models of five bacteriocins (Pediocin AcH, Mesenterocin Y105, Muditicin, Planatricin 423 and Sakacin G) (Fig 24-28). To the best of our knowledge these structures have not been reported earlier. The accuracy of the structures was evaluated using errat software (Verison 2.0) summarized in Table 19.

5.3.2 Bacteriocin modelled structures

FIG 28: Modelled structure of Pediocin Ach

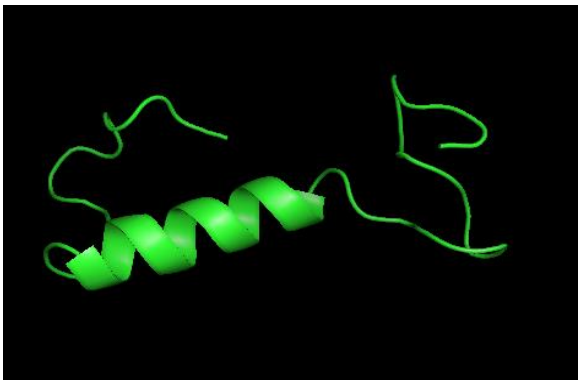


FIG 29: Modelled structure of Sakacin G

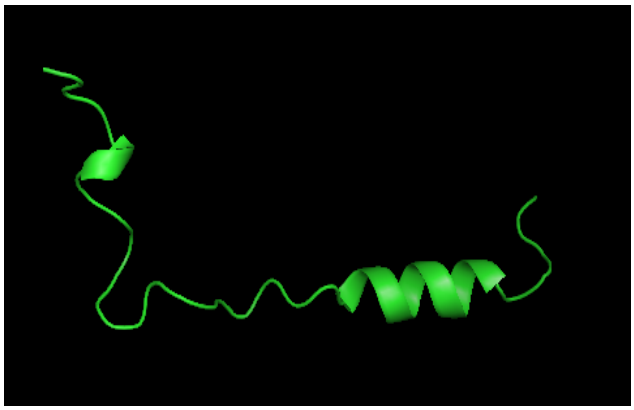


FIG 30: Modelled structure of Mesenterocin Y105

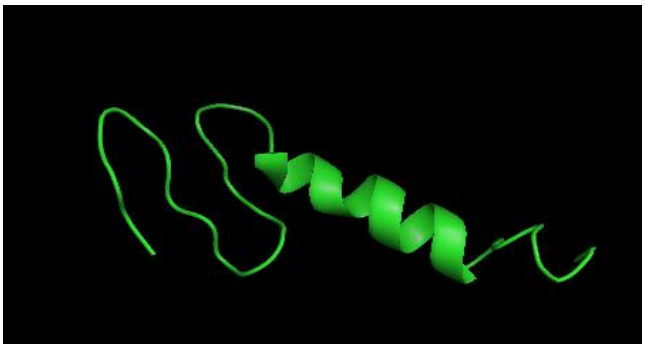


FIG 31: Modelled structure of Muditicin

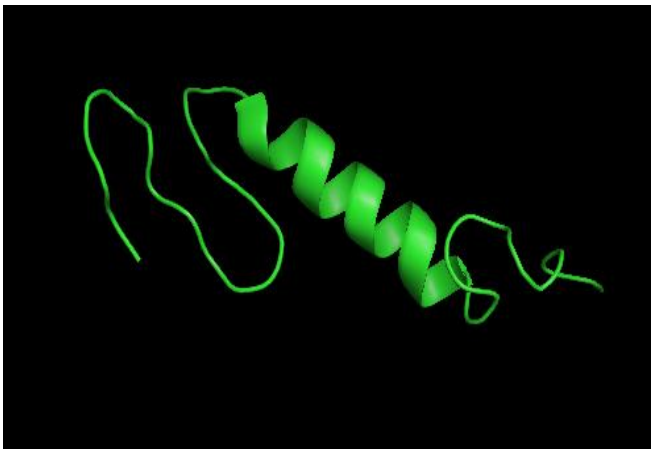
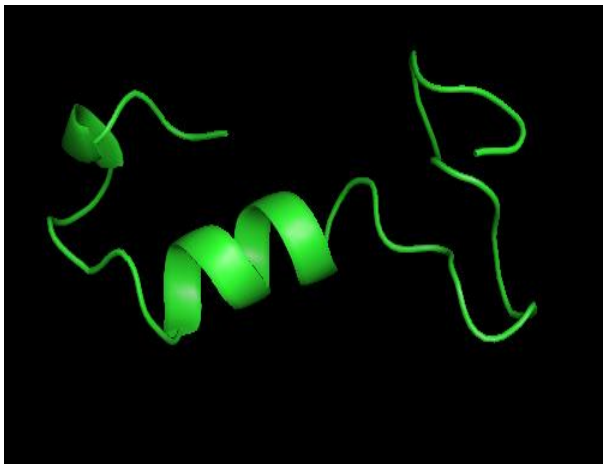


FIG 32: Modelled structure of Plantaricin 423



5.3.3 Errat efficacy

TABLE 20: Bacteriocins with the accuracy scores (percentage)

S. No	Bacteriocin	Errat accuracy score (%)
1	Pediocin AcH	80.00
2	Mesenterocin Y105	86.025
3	Munditicin	94.118
4	Planataricin 423	84.615
5	Sakacin G	100

The quality of the protein models is extremely important for the possible applications. As per the errat score, Sakacin G has the highest accuracy of 100%. The model generated were further analyzed by bioinformatics tool, Conserved Domain Search, and were found to have the same structural domain (PFAM 01721) confirming they all belong to the class(IIA) bacteriocin. The errat percentage signifies how good a modelled structure is. Since, this percentage for Sakacin G modelled structure is 100% we can say that this is the best possible modelled structure. And also only the structures with errat value of more than 80% have been used for the further studies which are currently in process.

6. DISCUSSION

6.1. Lactic Acid Estimation

Organic acids including lactic acid have been reported to show anti-microbial properties against many strains mainly of the gram negative genera (Alkami *et al*, 2000). It is believed that they act by affecting the permeability of the outer membrane of these bacterial stains hence affecting their normal functionality. Lactic acid has been attributed to cause the death of various microbial species (Midolo *et al*, 1995, Linngren *et al*, 1999). The most important aspect of the applicability of this kind of lactic acid inhibition as anti-pathogenic agent is its food grade and generally regarded as safe (GRAS) status. This has led to the utilization of this inhibition activity in various applications in food related systems (Lewis *et al*, 1991, Lewy *et al*, 2004, Schillinger *et al*, 1989). And all these reasons support the study of the activity of the applicability of these against entero-pathogens like *E.coli* and *S.typhii*. To the best of our knowledge this exact work has not been carried out by any other research group as yet.

A parallel experiment was setup between the growth of *Lactobacillus* species and the production of lactic acid. Lactic acid production was estimated after every 24 hours. According to the growth kinetics of the species, maximum growth occurred at 72 hours, therefore it was possible that lactic acid production was also maximum at that point. Lactic acid kinetics shows that among the four species maximum lactic acid is produced by *Lactobacillus delbrueckii*. At this point we could not be sure as to lactic acid is responsible for the inhibition or not. We had just established that the maximum production of lactic acid could be seen in *Lactobacillus delbrueckii.* Now, to study the correlation between the lactic acid production and the inhibition caused by it a well diffusion assay was set up to support the hypothesis that the inhibition is directly proportional to the lactic acid content. This is confirmed by the diameter of the zone of inhibition measured on the plates. It can be inferred that *Lactobacillus delbrueckii* has a greater amount of anti-pathogenicity as compared to others.

6.2 Bacteriocin estimation

Bacteriocins exhibit bacteriocidal and bacteriostatic activities usually against closely related species. These find applications in food preservation due to their efficient action against spoilage organism *Listeria monocytogenes.* It is also well known that, the lactic acid starter cultures of fermented foods display numerous antimicrobial activities which is mainly

because of their ability to secrete a variety of bacteriocins. Another significant point of interest is their antimicrobial activity against pathogenic microorganisms which have been reported by several workers. (Lewus *et al*,1991, Jones *et al*,2008, Ryan *et al*,1998). Through our research and through the use of the BACTIBASE database we have been able to find out that these show a wide range of effect against *Lactobacillus, Leuconostoc, Pediococuss, Listeria monocytogenes, Listeria innocua, Listeria invanovi, and Clostridium botulinum, Carnobacterium, Enterococcus, Lactobacillus, Leuconostoc* and *Pediococcus*. This has led us to develop the hypothesis that bacteriocins could be used as probable anti-pathogenic agents. Thus we started by setting up the experiment for the purified bacteriocin against entero-pathogens. An important point to be noted here is that these could be used against pathogens derived from the focal matter of patients suffering from various diseases. This is a very interesting observation since we have in this been able to prove that these bacteriocins are effective against disease causing organisms.

The bacteriocin activity was checked by the method of co-incubation wherein the Lactobacilli and the pathogen were allowed to grow on the same plate at the same time. The results obtained showed an activity of bacteriocins against these pathogens. The best results were obtained in *Lactobacillus rhamnosus.* Through this experiment and putting in parallel the results obtained from the experiment carried out with lactic acid we can say that these lactic acid bacteria show an activity against entero-pathogens. On observing the zone of inhibition, we can also conclude that *E.coli* (MTCC 723) is the most likely pathogen against which the *Lactobacillus* species show the anti-pathogenic activity. Since these are food grade bacteria they have a higher applicability as compared to the other bacterial strains. Generally regarded as safe (GRAS) these bacteriocins can further be used for the fortification of food matrices. As mentioned above the functional food aspect is growing by the day and in the future the applicability would increase more. With the help of this study we can establish that these lactic acid bacteria would be curative and help in fighting against the pathogenic bacteria. Also, since in both the experiments *Lactobacillus rhamnosus* and *Lactobacillus delbrueckii* show the best results it can be established that these have a very high efficacy against entero-pathogens. Also through the bioinformatics based approaches since we have been able to fins structures which were earlier not available we can say that these can be used for the analysis in the food matrices and hence we can obtain a food matrix with a very high efficacy.

6.3 Modelled structures

The quality of the protein models is extremely important for the possible applications. As per the errat score, Sakacin G has the highest accuracy of 100%. The model generated were further analyzed by bioinformatics tool, Conserved Domain Search, and were found to have the same structural domain (PFAM 01721) confirming they all belong to the class(IIA) bacterocin. The presence of same structural domain suggests that these bacteriocins may have a similar functional

application. One of the best studied representatives of Class IIA is Pediocin AcH so much so that the class IIA is often also called as Pediocin-like bacteriocin (Finland *et al*,2005). Pediocin AcH is a very effective food preservative (Cleaveland *et al*,2001 ,Holzapfei et al, 1995, Bhunia et al, 1999). The various food matrices where Pediocin AcH has been applied are –Munster cheese (a variety of smear soft cheese) (Saiid *et al*,1998), chicken, smoked salmon (Aesan *et al*,2003). Based on the above results of protein structure modelling, we further conclude that the other four bacteriocins might also have similar functional activity and stability in the above mentioned food matrices. Pediocin AcH has an anti microbial spectrum against *Lactobacillus, Leuconostoc, Pediococuss, Listeria monocytogenes, Listeria innocua, Listeria invanovi, and Clostridium botulinum, Carnobacterium, Enterococcus, Lactobacillus, Leuconostoc and Pediococcus*. It may be noted that the anti-microbial spectra of Plantaricin 423 and Sakacin G is not yet known. Considering the results of the modelling data presented in this study, it is indicated that these two may have the similar anti-microbial spectra. Further experimental studies would give an insight to the functional aspects of these bacteriocins.

7. CONCLUSION

Through this study we have been able to establish that the *Lactobacillus* species due to the secretion of lactic acid and other antimicrobial compounds like bacteriocins show a great deal of activity against entero-pathogens. The most interesting observation was that the selected *Lactobacillus* strains showed activity against the pathogenic bacteria isolated from the samples taken from patients suffering from various entero-pathogenic disorders. Also through the bioinformatics based approaches we have been able to find out structures of bacteriocins which were earlier not known available. These structures are now further being used to study the interactions of these proteins with the various food components. Bacteriocins have been used as food additives from a long time but the problem comes when they have very low efficacy in these food matrices. Through this study the solution we can say we can with time design a fortified food material with a very high efficacy and having a wide range of activity against various enteropathogens.

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