CHARACTERIZATION OF PROBIOTIC CULTURES FROM TRADITIONAL FOODS OF HIMACHAL PRADESH AND THEIR EFFICACY AGAINST COLONIZATION BY CRONOBACTER SAKAZAKII IN CAENORHABDITIS ELEGANS MODEL SYSTEM

Thesis submitted in fulfillment for the requirement of the Degree of

Doctor of Philosophy

By

KAVITA SHARMA

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

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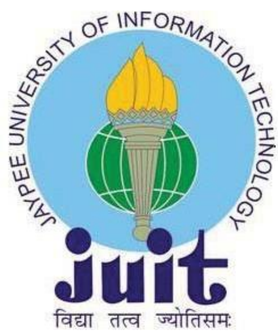

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Waknaghat, P.O.

Dumehar Bani, Kandaghat, Distt. Solan – 173234 (H.P.) INDIA

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Characterization of probiotic cultures from traditional foods of Himachal Pradesh and their efficacy against colonization by *Cronobacter sakazakii* in *Caenorhabditis elegans* model system"**, submitted by **Kavita Sharma** at **Jaypee University of Information Technology, Waknaghat**, **India** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Supervisor **Date:**

Dr. Gunjan Goel*

Associate Professor
Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology
Waknaghat, Solan, H.P. India-173234

*Currently working as:
Associate Professor
Department of Microbiology
School of Interdisciplinary and Applied Sciences (SIAL)
Central University of Haryana
Mahendergarh, Haryana, India-123031

ABSTRACT

Fermentation of raw substrates by microorganisms enhances the nutritional value of food by enhancing the nutrient contents, digestion of protein and fibers, elevating bioavailability of micronutrient and also decomposes anti-nutritional determinants. Therefore, fermented foods can serve as a reservoir of beneficial bacteria known as Probiotics which are the 'live microbes' capable of health-related advantages when given in appropriate doses to the host. There are scanty reports on diversity and role of these beneficial bacteria in traditionally fermented foods in Himachal Pradesh, which is a state in the North Western Himalayas. Therefore, this study targeted the identification of probiotic cultures used as starters in preparation of these foods, their characterization and efficacy to limit the colonization by pathogens using challenge studies in *Caenorhabditis elegans*. A total of 150 samples (fermented cereal-based dough and fermented dairy samples) were analysed for isolation of probiotics from different places and districts of Himachal Pradesh and were examined *in vitro* for their potential probiotic attributes. Out of 150 samples 101 isolates were obtained on deMan Rogosa Sharpe (MRS) agar plates having opaque, white and creamish colonies which were further identified as Gram-positive rods, cocci, facultative anaerobes and catalase negative. Subjecting to safety attributes depicting hemolytic activity on sheep blood agar plates, out of 101, 51 isolates were found to be non-hemolytic which were further screened for the passage of GI tract conditions. Out of 51 isolates 15 isolates were survive under simulated gastric and intestinal fluids. The isolates were identified targeting 16S rRNA sequences belonging to group of Lactic Acid Bacteria (LAB). The following isolates were identified using the 16S rDNA region: *Brevibacillus thermoruber* (*n* = 3), *Lactobacillus gastricus, Brevibacillus aydinogluensis, Enterococcus sp., L. paracasei, Weisella confuse*, *Lactobacillus fermentum* (n=3), *L. plantarum* (n=2), and *Pediococcus acidilactici* (n=2). These isolates were further investigated for *in vitro* and *in vivo* probiotic attributes. The aforementioned strains exhibited resistance to highly acidic conditions (pH =2.0) alongwith 1% digestive fluid salts, and pancreatin (1mg/L). The maximum survival to these simulated gastric and intestinal conditions was observed in *L. paracasei* CD4 and *L. gastricus* BTM7. The highest scores for other probiotic attributes such as cell autoaggregation, cell surface hydrophobicity, and adhesion to Caco-2 cell line were observed in most of the isolated strains.

Following evaluation of probiotic strains using in vitro tests, further the health promoting effect of these probiotics was determined by feeding these cultures to in vivo model *Caenorhabditis elegans*. The impact of feeding on mean life expectancy, pharyngeal pumping, regular reproductive behavior and chemotactic conduct in the concern for choice of index was conjointly assessed. Feeding these probiotics did not have any negative effect on physiology of the worm such as pharyngeal pumping, reproduction etc, moreover, the strains displayed colonization and adherence within the gut of the worm and expanded life expectancy like five days when compared with control *E. coli* OP50. Further, the investigation was carried out on the antimicrobial activities of these strains against *Cronobacter sakazakii* in *Caenorhabditis elegans*. The probiotic cultures exhibited varying degree of antimicrobial activities against the *C.sakazakii*. The Cell free supernatant (CFS) of *L. gastricus* BTM7 and *L. plantarum* K90 exhibited maximum antimicrobial activity against *C. sakazakii*. A 40 µl CFS of probiotic isolates were found to inhibit the biofilm formation by *C. sakazakii*. The pathogen *C. sakazakii*, resulted in complete killing of the worm in five days as compared to standard food of
E. coli OP50 with a extended mean life span (MLS) of 16 days. The pathogen also resulted in impaired pharynx, distorted intestine, poor vulval growth and internal hatching of the eggs in the worms. However, the probiotic interventions at different stages limit the colonization and infection by the pathogen. The protective effects of probiotics against the infection of *C. sakazakii* in *C. elegans* were determined via competitive exclusion assays which included inhibition, competition and displacement. Among these, the competition and displacement assays depicted increase in MLS of the worm by two to three days. A pretreatment with probiotic isolates was found to result in better protection of the worm against infection with *C. sakazakii* by extending the life of the worm.

The study suggested that traditional indigenous fermented foods are reservoir of probiotic cultures portraying different probiotics and functional attributes. The preconditioning with probiotic lactic acid bacteria can be taken as an effective measure to overcome the invasion and colonization by the pathogens. However, the results need to be further validated using in vivo mice models targeting immune-modulatory responses during probiotic interventions.

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DECLARATION

I hereby declare that the work reported in Ph.D. thesis entitled **"Characterization of probiotic cultures from traditional foods of Himachal Pradesh and their efficacy against colonization by *Cronobacter sakazakii* in *Caenorhabditis elegans* model system"** submitted at **Jaypee University of Information Technology, Waknaghat, India** is an authentic record of my work carried out under the supervision of **Dr. Gunjan Goel.** I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis.

Kavita Sharma **Date:**

Enrollment No. 146551
Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology
Waknaghat, Solan, H.P. India-173234

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
CFU	Colony Forming Unit
CGC	Caenorhabditis Genetics Centre
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribo nucleic acid
dNTPs	Deoxynucleotide Triphosphates
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
G	Gram
H_2O_2	Hydrogen peroxide
K_2HPO_4	Di- potassium hydrogen phosphate
Kg	Kilogram
L	Litre
LAB	Lactic Acid Bacteria
M	Molar
Mg	Milligram
mL	Millilitre
mM	Millimolar
MRS	De man Rogosa Sharpe
N	Normal
N	Mean
NGM	Nematode Growth Media
Nm	Nanometre
OD	Optical density
P	Probability of error
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
r^2	Pearson correlation coefficient

SI	Small intestine
sp.	Species
SPSS	Statistical package for the social sciences
TSB	Tryptone Soya Broth
XLSTAT	Statistical software for excel

Chapter 1

Introduction

1.1 Fermentation of food and significance

The process of fermentation has been used from time immemorial for enhancing the longevity of food products across the world. It primarily includes utilization of microbes or microbial enzymes for transforming simple components, such as carbohydrates, into products of high significance (organic acids) [1]. Fermentation enriches the nutritional value of food by the means of vitamin biosynthesis, production of essential amino acids and proteins, enhances digestion of protein and fibers, elevates bioavailability of micronutrient and also decomposes antinutritional determinants. The fermentation of food aids in lowering the concentration of toxic substances like aflatoxins and cyanogens and results in production of antimicrobial substances like acetic acid, bacteriocins, carbon dioxide, hydrogen peroxide and ethanol, which confers protection against food-borne pathogens and thereby enhances the food safety. Furthermore, fermentation of food leads to addition of different types of flavors, textures and fragrances [2].

Over the past several decades, fermented foods have gained popularity across the globe and in few regions, they constitute the major portion of daily diet of uncountable number of people. Native fermented foods like bread, cheese and wine have been produced and consumed from time immemorial and have gained high cultural and traditional significance. Considering the traditional importance and economic benefits associated with fermented foods, fermentation has been hugely leveraged in developing nations for producing food and beverages. The native fermentation procedures have gained considerable amount of attention from researchers as well as food policy makers as a part of food safety approach. Recent technological advancement in the field of biotechnology and microbiology has opened further avenues for upgradation of native fermentation procedures, their optimization and enhancement of the yield of final product and furthermore have facilitated effective utilization of commonly available agricultural and horticultural products and the derived waste products as substrates for producing fermented food products [3][1].

Since a long period of time, the process of fermentation has been used as a strategy for food preservation and improvising the longevity, taste, composition and nutritional value of food, [4] however, in recent past the consumption of fermented foods possessing live microbes has been widely acknowledged as a significant diet-related strategy for enhancing the health of human beings [5]. From the available literature on fermented foods, it can be inferred that ample number

of investigations have been carried worldwide to ascertain the physiological benefits of fermented foods, for example, enhancement of nutrient absorption and improving the health of digestive system [6] [7].

Lactic acid bacteria, such as *Lactobacillus*, *Streptococcus*, *and Leuconostoc* are majorly used for fermentation of food along with several fungal and yeast species. Fermented foods produced on an industrial scale often serve as a good source of probiotic cultures, however, knowledge about the probiotic microflora of different fermented foods remains unexplored to a huge extent [8].

1.2 Traditional fermented foods of Himachal Pradesh

The Himalayan region is described by assorted ethnic groups, which have built up their own tradition depending on accessible resources, resulting in cultural as well as biological differences. Locally fermented foods are extremely famous in the tribal and rural zones of Himachal Pradesh [9]. These have gained recognition and the associated conventional fermentation processes have been examined extensively by the investigators. Few well known fermented food items and beverages have been examined for assessing their microbiological qualities. The fermneted items that exclusively found in the tribal and rural areas of Himachal include Bhaturu, Siddu, Chilra, Sepubari, Sura, Chhang, Lugri, Daru, Manna, Marchu, Bagpinni, Seera, Dosha, Angoori and Behmi. Apart from conferring nourishment, these fermented items, e.g., Bhaturu, forms the major part of staple diet widely in rural regions of Kullu, Kangra, Mandi and Lahaul and Spiti of the state of Himachal Pradesh whereas the remaining fermented items are consumed during traditional celebrations, weddings and other special events [10]. A portion of the locally fermented food items occupy a significant position in religious and socio-social activities of rural individuals in Himachal. These food items are exceedingly nutritious, effectively edible, classy, simple to protect and are mainstream among the rural people. Fermented food items have not only established their nutritional importance but have also substantiated their probiotic relevance and their ability to confer protection against various ailments.

Locally used starter cultures like Phab, Treh and Malera are inoculated to obtain the desirable fermented food item. It has been found from various microbiological examinations that species of *Saccharomyces cerevisiae* is primarily leveraged in fermentation process in association with *Candida sp., Leuconostoc sp.* and *Lactobacillus sp.* Furthermore, the concentration of ethanol produced during fermentation of food has likewise been investigated. The locally fermented food items and beverage constitute the major portion of daily eating routine of the general population residing in the tribal regions of Lahaul and Spiti, Kinnaur, Chamba as well as rural zones of Kullu, Shimla, Mandi and Kangra areas of Himachal Pradesh. Extensive variety of locally fermented food items are produced and consumed in Himachal Pradesh. The technical knowledge pertaining to the conventional procedures associated with the generation of fermented items has been passed from one generation then onto the next. The crude materials required for fermentation are derived from locally available resources and very simple and easy to use equipments are utilized by the locals for carrying traditional food fermentation procedures [11].

1.3 Fermented foods as reservoirs for probiotics

Live microbes interact extensively with the surrounding environment by utilizing the constituents of the substrate for carrying their metabolic activities. Therefore, it can be inferred that the chemical composition of the substrate or the growth medium is of principal importance in order to facilitate the metabolic activities of the microorganisms. Basic factors that are of great significance include the available concentration of the carbohydrates, the level of hydrolysis of proteins present, which decides the essential amino acid availability, and the constitution and level of hydrolysis of drain lipids, which helps to estimate the available short-chain unsaturated fats. Furthermore, the proteolytic and lipolytic characteristics of probiotics might be imperative for further breakdown of proteins and lipids. These two characteristic features are potent enough to significantly impact the flavor of the fermented food products [12].

While manufacturing probiotic fermented products it is imperative to determine the level and type of interaction between probiotic microbes and starter culture used. Although, only a couple of studies have reported about such interactions, yet both synergistic and antagonistic interaction between various starter culture have been substantiated. For instance, the yogurt culture is

identified by a photosymbiotic interaction between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. bulgaricus. This synergism as exhibited by a quickened and efficacious fermentation of the milk and proliferation of live culture microbes is actually dependent on the type of strain used for the fermentation process [13]. Furthermore, antagonism is another type of microbial interaction found during fermentation of foods. Antagonistic interactions result from compounds causing inhibition or inactivation of the starter cultures used and thus interfere with the fermentation process. In particular, antagonistic effects are caused majorly by bacteriocins, which are peptides or proteins displaying anti-microbial features. Apart from this, other antagonistic effects include production of H_2O_2, benzoic acid, biogenic amines and lactic acid [14][15].

The level of the interaction taking place between probiotics, substrate composition and the starter cultures depends extensively on the time when probiotics are introduced to the fermentation process, which can be before or during the process of fermentation. However, it has been observed that interaction is very low when probiotics are incorporated promptly before or even subsequent to cooling underneath 8°C as the metabolic activity of starter cultures and probiotics gets radically lessened at these temperatures. However, if the fermentation process is extended for longer duration then even minimal interaction may indicate quantifiable outcomes.

1.4 Probiotic cultures, characteristics and importance

Probiotics are characterized as 'live microbes’ capable of granting health-related advantages when given in appropriate doses to the host' [16]. Alternately, probiotics have been characterized as live microbial food supplements that help in providing health advantages to the host by enhancing the intestinal microflora. Probiotics were initially used to enhance the health of the human beings as well as animals by the means of the intestinal microflora modification. Various strains of *Lactobacillus* and *Bifidobacterium* have been acknowledged for their potential to minimize incidents of gastrointestinal infections or cure them completely [17]. Several advantages of probiotic utilization comprise of enhancement of intestinal wellbeing by regulating the gut microflora, and activating and enhancing the immune system, producing and improving

the bioavailability of nutrients, diminishing side effects of lactose intolerance, and decreasing the danger of certain different ailments (Fig 1.1) [18].

Early investigations carried on the probiotics were majorly directed towards prevention and treatment of gastrointestinal ailments [19]. Modification of gut microflora has been related with upgraded danger of ailments and thus it can be deduced that regulation of an unequal indigenous microflora forms the base for probiotic treatment [20]. Likewise, the advancement of adjuvant or other equally effective treatments dependent on bacterial substitution is gaining more importance in view of abrupt surge in the population of antibiotic-resistant pathogenic strains and harmful impact of antibiotic treatments on useful microflora, which contributes towards the danger of developing infection [21].

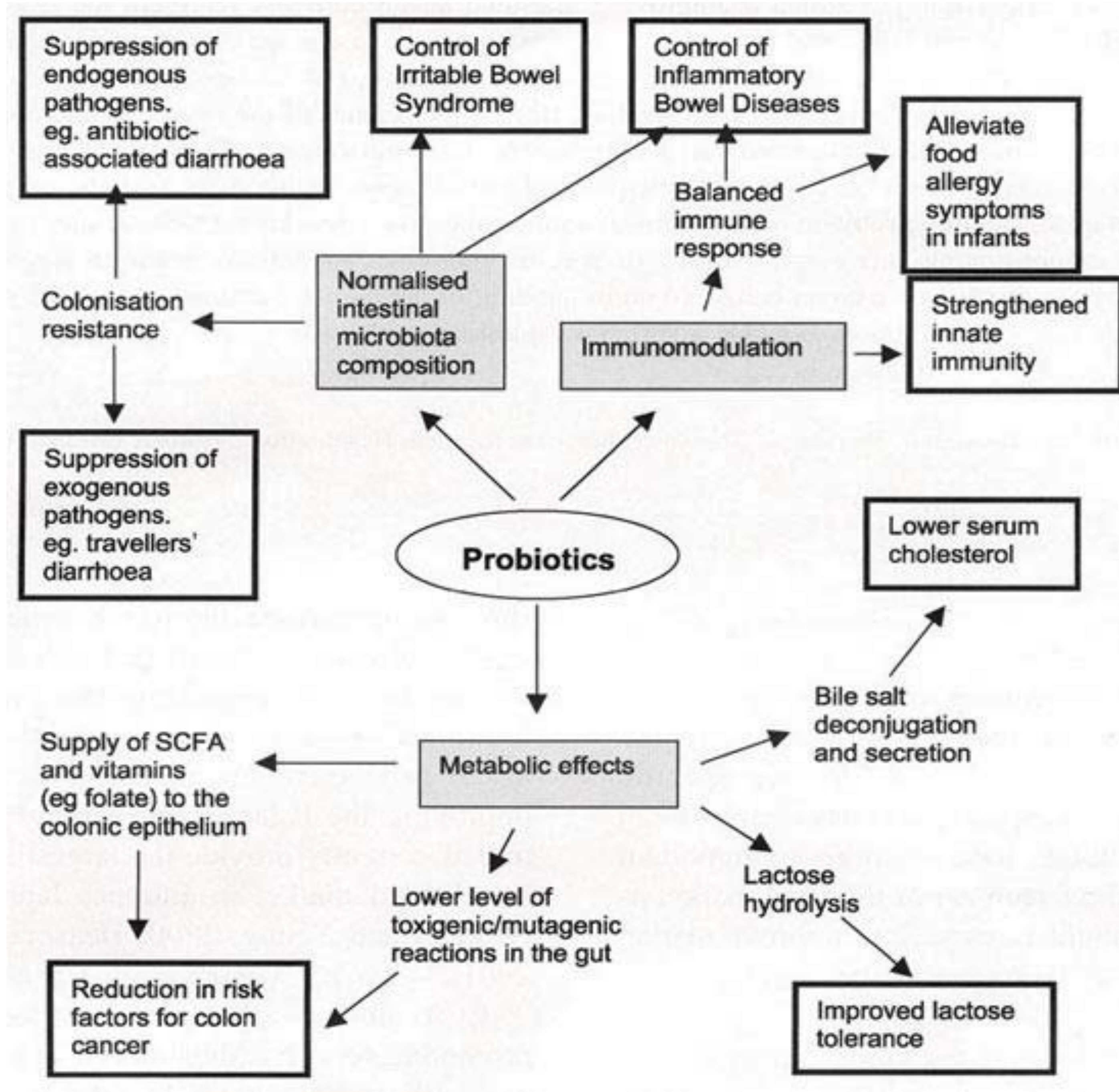


Figure 1.1: Proposed health benefits of Probiotics [22]

In any given case, the utilization of probiotics ought to be additionally examined for their advantages and conceivable symptoms. As the awareness about intestinal microflora, nourishment, immunogenic responses, and genes involved in regulating health and diseases has enhanced in the previous years, such type of data could absolutely aid in developing new probiotic strains exhibiting disease-specific responses and could likewise encourage the comprehension of when to utilize probiotics and how they influence a particular ailment. In any case, it is imperative that the probiotic strains selected to be administered to humans should be at first medically tested on animals to verify its appropriateness, health related advantages, and advantages of probiotics for human utilization and production of functional foods [23].

It is of most extreme significance that the probiotic strain survives the surrounding conditions of the location where it is expected to play an active role. In order to attain highest possible activity, the strain ought to have the capacity to multiply and colonize at this particular area. Furthermore, it needs to be ensured that the probiotic strain should not elicit any anti-immunogenic response, i.e., the strain should not exhibit any kind of pathogenicity, hypersensitivity, mutagenicity or carcinogenicity [24][25]. Probiotics chosen for humans ought to meet the required food safety standards and should possess no or very low risk of developing any kind of ailment (Fig. 1.2).

It is imperative to consider several characteristic features while selecting a probiotic culture for human use. The selected probiotic culture needs to be ideally derived from humans, must have the capacity to survive and develop in the *in vivo* conditions of the target site of administration, and accordingly should have the capacity to endure low pH and high concentration of bile acids. For ensuring their effective utility in foods, the probiotic utilized should be able to withstand the food production techniques used in industries. Furthermore, the food items possessing the probiotic bacterial cultures should not interfere the inherent features of food [23].

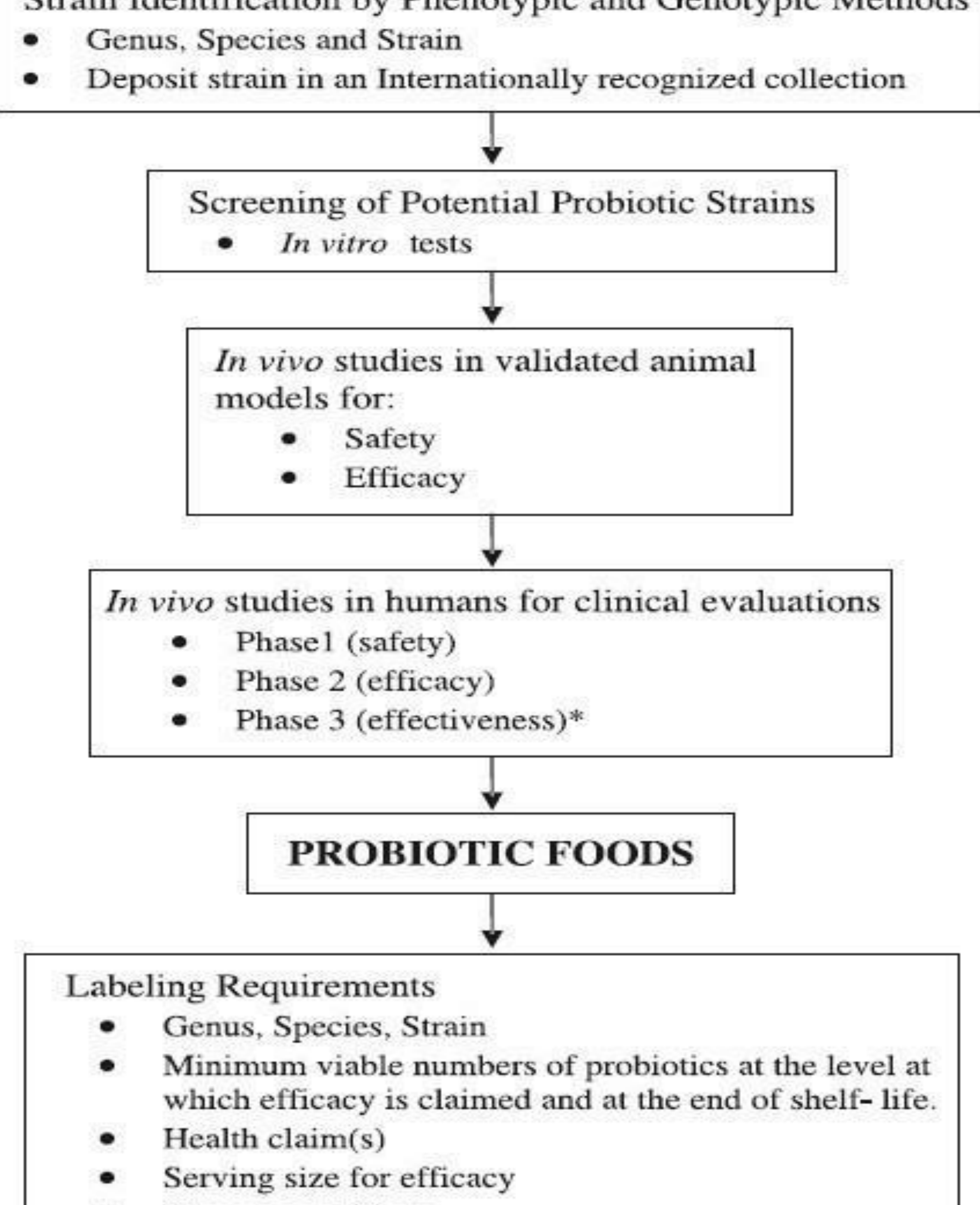


Figure 1.2: Protocol for evaluation of probiotics for applications in food as provided by DBT-ICMR

1.5 Role of lactic acid bacteria as Probiotics

The group, Lactic acid Bacteria (LAB) constitute gram positive, acid-tolerant/ resistant and majorly non-sporulating bacterial species that breakdown complex carbohydrate into simpler molecules by producing lactic acid. Lactic acid bacterial species differ from each other based on their nutritional requirements which also determine their site of proliferation. The lactic acid bacterial species considered as probiotic include *Lactobacillus, Pediococcus, Streptococcus* along with peripheral species such as *Enterococcus* and *Weisella* [26].

LAB gained commercial importance as probiotic as they have been declared safe for consumption, are naturally found in wide range of foods and confer health benefits to the host by eliminating harmful microflora. They accomplish this by producing antimicrobial compounds, competing for adhesion receptors and nutrition and eliciting host immune response [27]. The antimicrobial compounds produced by LAB include lactic acid, peroxide, bacteriocinsand bacteriocin-like inhibitory compounds (Fig. 1.3). The LAB prevents adhesion of harmful microbes by competing for the attachment sites and forming a protective layer around the epithelial cells. LAB are highly fastidious and compete for range of nutritional substances. Availability of a specific type of nutrition enables LAB to outgrow other microbes. When administered in appropriate concentration, LAB lead to elicitation of immune response by producing helper cells and suppressor cells and triggering differentiation of lymphocytes [28].

1.6 *C. sakazakii*: the target pathogen

Cronobacter achieved the status of genus just few years back and was formerly known as *Enterobacter sakazakii*. Cronobacter species are becoming prevalent as foodborne pathogens and have been distinguished as the causative agent responsible for few episodes or sporadic instances of intense neonatal infections causing meningitis, septicaemia or necrotisingenterocolitis in newborn children [29]. The affected individuals regularly exhibit intense neurological disabilities, for example, hydrocephalus, hydrocephalus, quadriplegia, brain abscess and impeded neural advancement [30]. The environmental reservoir of *C. sakazakii* is not evidently understood however, the epidemiological studies suggested reconstituted powdered infant formula (PIF) as the possible source of resrvoir and transmission of the pathogen [31].

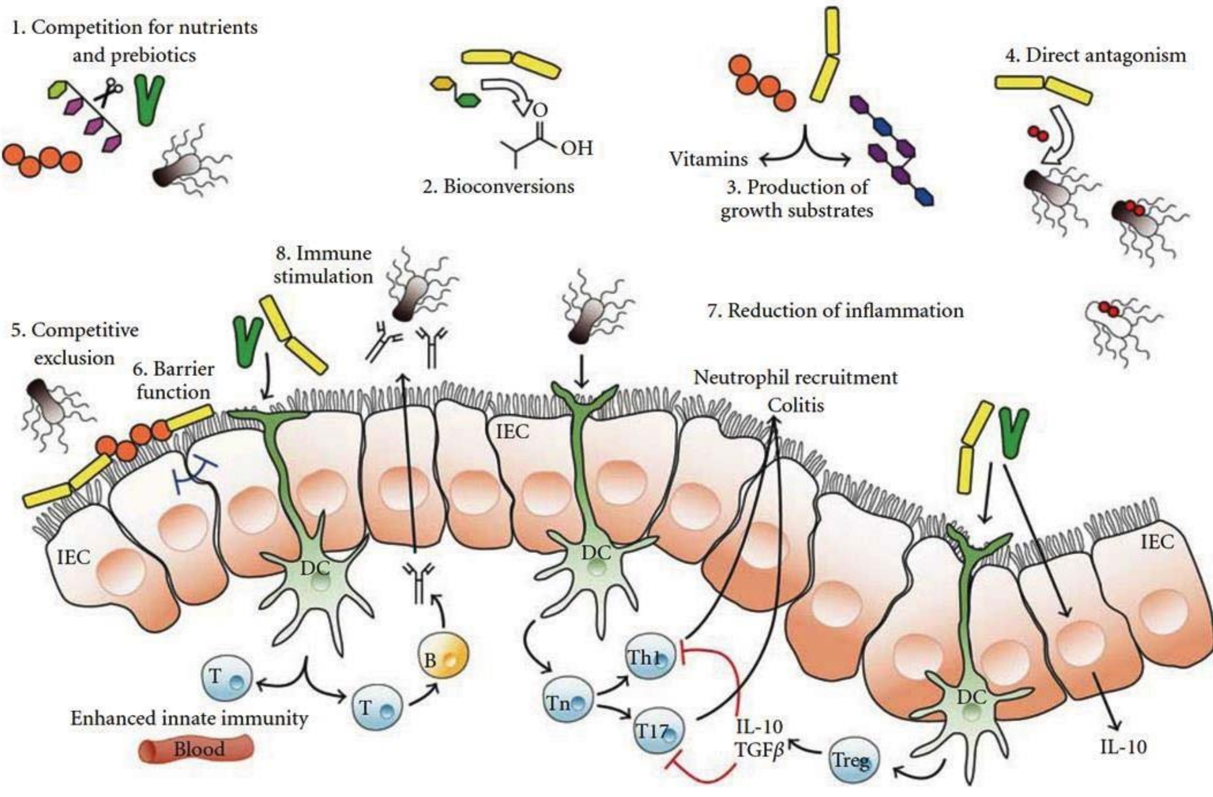


Figure 1.3: The proposed action mechanism and beneficial effect of lactic acid bacteria in intestinal epithelial cell (Source from www.customprobiotic.com).

"(1) competition for nutrients (2) production of antimicrobial compounds (3) production of growth prompting substrates, for example, EPS or vitamins, for other bacteria, (4) antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation (8) stimulation of innate immune response (by unknown mechanisms). IEC: epithelial cells, DC: dendritic cells, T: T-cells"

From the available literature it could be inferred that majority of the investigations were carried for identification and classification of *Cronobacter* species with very little focus on their pathogenicity. Investigators have posited about several defense mechanisms adopted by *Cronobacter* to support their survival, which includes development of biofilm layer, attachment to hydrophilic and hydrophobic sites, synthesis of antimicrobial compounds and quorum sensing [32]. Among these, biofilm production accounts majorly for its pathogenicity (Fig. 1.5). The biofilm production take place with the aid of different cellular parts like flagella, fimbriae and EPS. *C. sakazakii* defend themselves by forming biofilm on the surfaces that may come in contact with the food during various procedures or inside the cells of the host, which helps them to thrive under stressful situations. This way *C. sakazakii* pose threat for food contamination and promote food-related ailments [33]. Our laboratory has previously reported the prevalence of *C. sakazakii* among different food and environmental commodities. The isolates expressed different virulent factors (Fig. 1.4) and were reported to form biofilm mediated by expression of acyl homoserine lactones (AHLs) as signaling molecules and genes encoding for cellulose synthesis [34][35]. Although probiotic cultures have been reported for their antimicrobial activities against an array of pathogens leading the gastrointestinal disorders, however very few studies have been conducted to check the efficacy of probiotics against *C. sakazakii*.

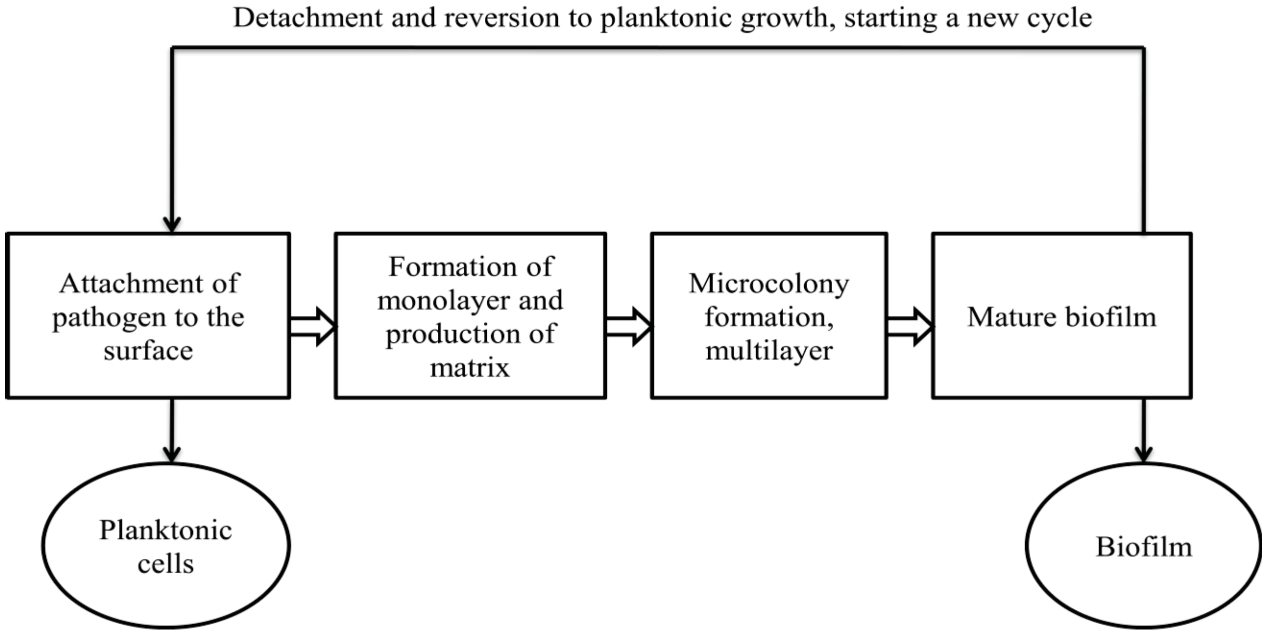
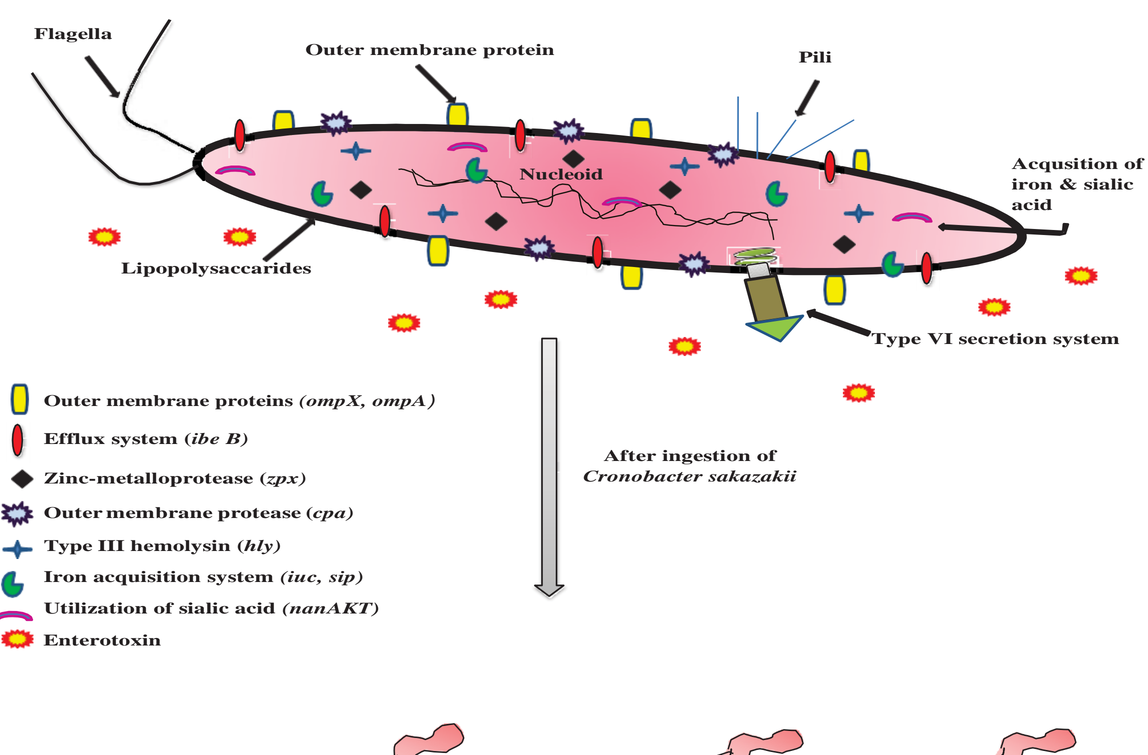


Figure 1.4: Biofilm formation by *C. sakazakii*



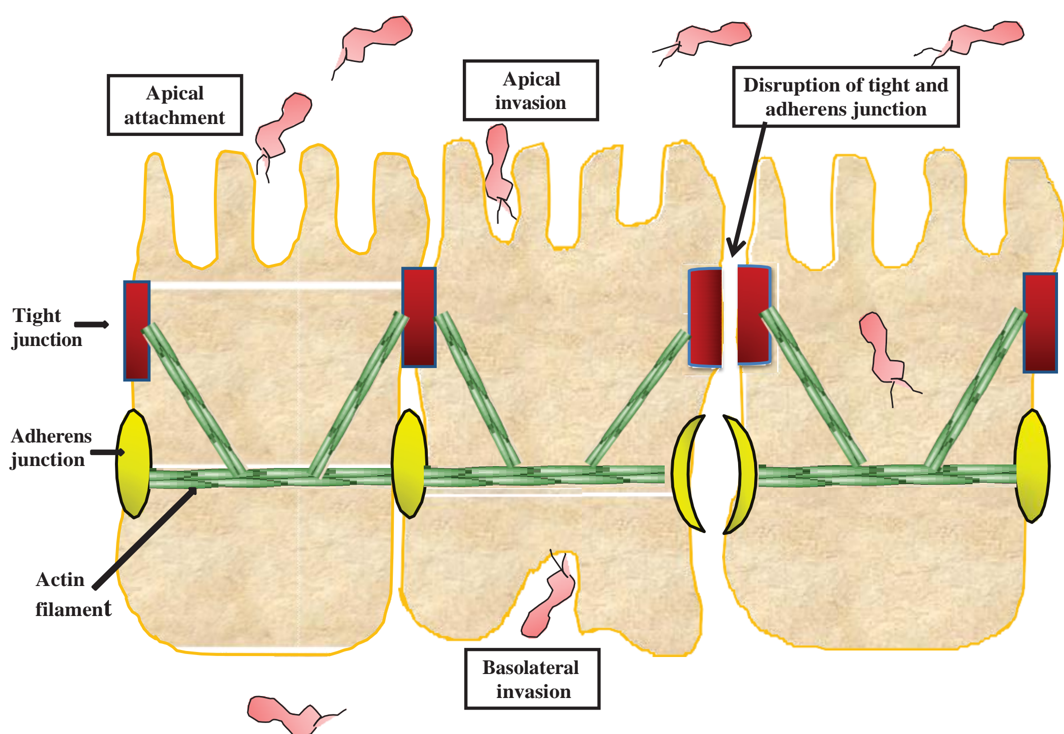


Figure 1.5: Suggested model for infection and pathogenesis of *Cronobacter sakazakii* [36]

1.7 Model system *C. elegans*: a suitable model for probiotic studies

The present lack of microbiome investigations pertaining to *C. elegans* is unforeseen, on the grounds that few attributes make this nematode appropriate for the exploratory examination of host-organism associations. Various advantages of using *C. elegans* as model system can be: It is easily subjected to genetic alterations. the nearness of microorganisms can be productively controlled utilizing the bleaching procedure, which is just made do by nematode eggs however no organisms, accordingly permitting development of nematodes under axenic or monoxenic conditions [37]. Third, *C. elegans* is transparent which facilitates easy microscopic examination of microbial accumulation inside the whole animal. Fourth, a few previously carried investigations advocate for considering *C. elegans* as an ideal model for studying microbial interactions. Taken together, C. elegans is a great exploratory model to efficiently break down the impacts of the microbiome on the host and the other way around. Because of these points of interest, *C. elegans* has been utilized widely to examine have pathogen communications, including for the most part bacterial pathogens (Fig. 1.6).

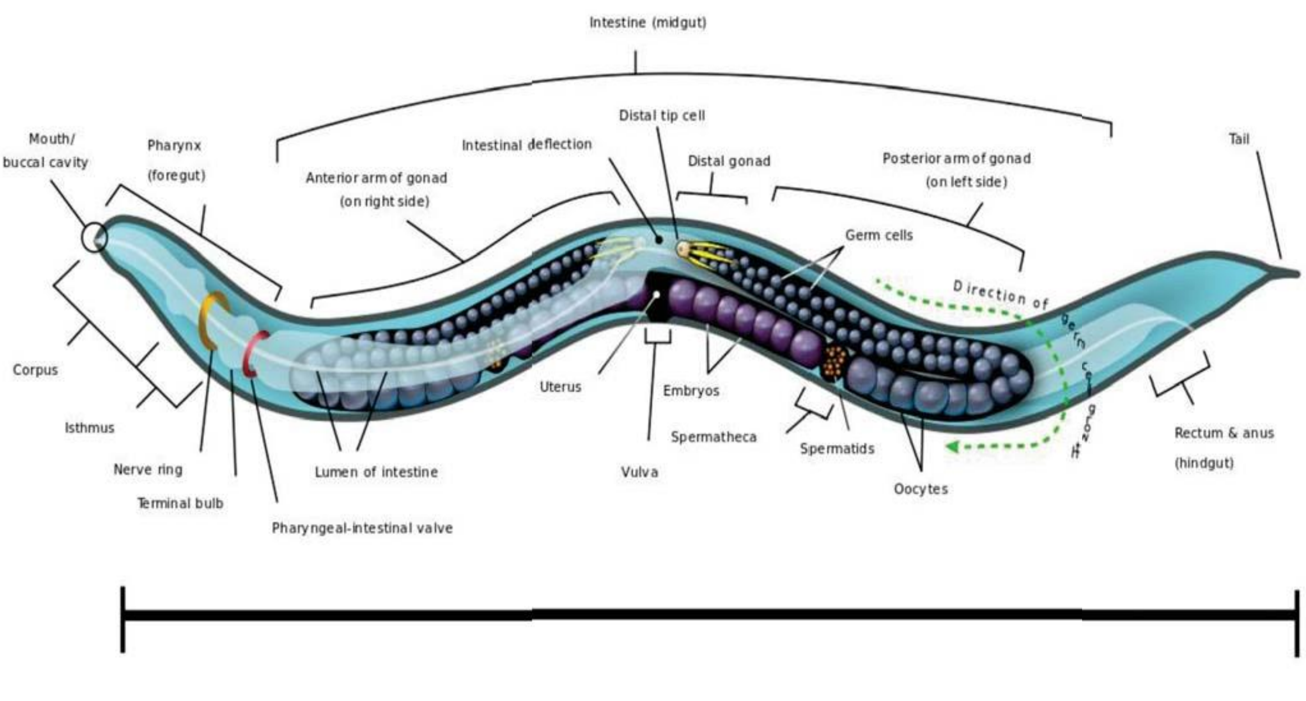


Figure 1.6: The anatomy of *C. elegans* [38].

Table 1.1: Comparison of most popular model organism and reason why model system *C. elegans* is a suitable model for probiotic studies

Saccharomyces cerevisiae	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Mus Musculus*	*Homo sapiens*
Single, free living cell and 3 micron in diameter	Multicellular animal, 1 mm long	Multicellular animal, 4 mm long	Multicellular animal. High homologue with human, 180 mm long	Multicellular animal, 5-6 feet tall
Reproduced by budding and doubles every 90 min	Lifespan: 2-3 weeks. Produce new generation in three days	Lifespan: 30 days at 29 °C Produce new generation in 10 days	Lifespan: 2 years, Produce new generation in 9 months	Lifespan: ~70 years. Produced new generation in 20-25 years
Its genome was sequenced in 1996	Its genome was sequenced in 1998	Its genome was sequenced in 2000	Its genome was sequenced in 2002	Its genome was sequenced in 2001
12 million base pair of DNA	99 million base pair of DNA	165 million base pair of DNA	3 billion base pair of DNA	3 billion base pair of DNA
6,000 genes and 31% homology with human	19,000 genes and 40% homology with human	13,600 genes and 40% homology with human	Most of the human genes have a counterpart in mouse	~20,000-25,000 genes

A wide assortment of bacterial pathogens, and in addition a few growths, can execute *C. elegans* or create non-lethal sickness indications in the worm. Few of the human pathogens under this category include Gram-negative as well as Gram-positive pathogens such as*, Pseudomonas, Escherichia, Salmonella, Klesbiella*; whereas Gram-positive microbes include *Enterococcus, Staphylococcus*, and *Streptococcus*; and the growth *Cryptococcus neoformans* [39]. Generally, microbial pathogens colonize the *C. elegans* digestive system and abatement its life expectancy. Some of them hold fast to the nematode fingernail skin, while others deliver poisons that slaughter *C. elegans* without a requirement for the live bacterial cells to specifically contact with the worm. *Coronobacter* sp., a human pathogen, can contaminate *C. elegans* at a disease rate that relies upon the bacterial collection inside the host and initiate the antimicrobial qualities in *C. elegans* [40].

1.8 Aims and Objectives

Considering all the associated health benefits of probiotic microbes, it can be inferred that they are highly significant as probiotics and thus need extensive investigation. The available literature indicates that majority of the probiotic microbes have been isolated from dairy products and thus additional research work is required to isolate advance probiotics that are relatively more efficient and tolerant towards starter cultures employed during fermentation of foods. Traditional fermented food is part of basic diet of majority of tribal and rural people residing in the state of Himachal Pradesh and it is likely that the fermented foods may contain some important probiotic cultures. Thus, this study will aid in adding more to the existing literature by isolating probiotics from fermented foods of Himachal Pradesh. The present study endeavors to characterize probiotic cultures from fermented foods of Himachal Pradesh and examine their efficacy against colonization by *Cronobacter sakazakii* in *Caenorhabditis elegans* model system.

Objectives

1. To isolate and identify bacterial cultures from traditional fermented foods of Himachal Pradesh.
2. To screen the bacterial isolates for probiotic attributes using *in vitro* methods.
3. To study the effect of selected isolates for probiotic properties using model system *C. elegans*.

4. To study the protective effect of probiotics against infection with *C. sakazakii* in *C. elegans.*

Chapter 2

To isolate and identify bacterial cultures from traditional fermented foods of Himachal Pradesh

2.1 Introduction

Indian traditional fermented foods are reviewed many times in terms of their indigenous ways of production and the role of beneficial bacteria most commonly, Lactic acid bacteria, in modulating their various properties such as organoleptic properties, preservative nature and adding to the nutritive value to the food [1]. The studies related to prevalence, diveristy and role of these starter lactobacilli in preperation and production of these fermented foods are very limited [2]. Himachal Pradesh, a state which is lying in the region of North Westerm Himalayas is exceptionally different over its topography, culture and history of productio of various fermented food products. The processing and production of these fermented foods is generally recognised from family households to traditional small microscale units only [3]. As stated before, these fermented food products are traditional and are dependent on the autothochonous or native microbiota, which makes these fermented products as an important source or reservoir of beneficial indigenous lactobacilli as starter cultures [4]. Probiotic cultures are non-pathogenic microorganisms, which when taken in an adequate amount, are reported exert a positive health benefit on host [5]. Till date, most of the probiotics belongs to the genera comprising group of Lactic acid bacteria (Lactobacilli, Bifidobacteria, Streptococci, Enterococci) and yeasts such as *Saccharomyces sp.* As these organisms are native or indigenous to the fermented foods therefore these are also generally regarded as safe (GRAS) [6]. Among these genera, LAB is engaged with the maturation of the fermentation and is predominant microflora of these fermented food products. These genera are known to have a fundamental job in protection of nutritive value of the food and limiting the growth of other spoilage or pathogenic microorganisms via production of various antimicrobial compounds such as organic acids, proteinaceous bacteriocins, hydrogen peroxide (H_2O_2), diacetyl and CO_2 [7]. Utilization of these probiotic rich products has been reported to be useful in combating different clinical conditions extending from irresistible ailment (looseness of the bowels, uro-genital contaminations and others), insusceptible framework balance and expanded protection from pathogens [8], moderating the development of colon tumor, lowering the cholesterol levels, treating hypersensitivities and furthermore helps in alleviating antibiotic associated diarrhea (ADD) by following up on the immune system of the host [9].

The inhabitants of different regions of Himachal district are known to process and prepare a wide variety of dairy or cereal based fermented food products have profited as far as by and large

wellbeing, in light of the related beneficial indigenous microorganisms [10]. Additionally, the milk based fermented products could serve as a good delivery vehicle for probiotics as these types of fermented products serve as a reservoir of diverse types of LAB. Scanty reports are available from Himalayan districts pertaining to isolation and probiotic based characterization of indigenous LAB from different traditional fermented food commodities [11]. Additionally, for utilization of these lactic strains as dietary aides, survival and colonization of these cultures during the digestive tract conditions is recommended. Keeping these lacunae in the studies, the objective of this research is centered around bioprospecting novel strains of bacterial cultures from various fermented and non– fermented food items and their assessment for probiotic characteristics.

2.2 Methodology

2.2.1 Isolation of bacterial cultures from food samples

- The sample collection was done by using sterile vials (50 ml) using standard microbiological protocols (Fig. 2.1).
- A total of 150 samples (fermented cereal based dough (50) and milk based samples (100)) were collected from different places and districts of Himachal Pradesh.
- An aliquot of 25 ml of sample was collected in each vial and was preserved under refrigeration till analysis.
- The samples were further screened for the isolation of LAB using serial dilution method and spreading was done on MRS agar (de Mann Rogosa Sharpe agar) (Hi-media Laboratories, Mumbai).
- The plates were allowed to absorb the contents and were incubated at 37ºC for 48 h for the growth of bacterial colonies [12].
- After incubation, the single colonies were picked carefully from each plate and again subcultured to check for the purity of the isolate. The pure colonies were maintained on agar slants and glycerol stocks and were maintained at -80ºC for future use.

- All the experiments were performed in triplicates in this study and the results were compared with *Lactobacillus rhamnosus* GG which is a commercialized standard probiotic strain and was procured from NDRI, Karnal.
- Preliminary examination of the isolates was done by analyzing cell morphology, Gram's staining and catalase test.

A. Curd **B. Butter Milk** **C. Milk**

D. Fermented Dough **E. Fermented Dough**

Figure 2.1: Different samples collected from fermented dairy and cereal based fermented products

2.2.2 Biochemical Characterization of the isolates

Carbohydrate fermentation by isolated cultures

The bacterial cultures are known to have the capability of fermenting different sugars [13]. The sugar fermentation test was carried out in the lab by using HiLacto Identification Kit, (Himedia Laboratories, Mumbai, India) (Fig. 2.2).

- The bacterial isolates were grown overnight in MRS broth and on the next day 50 µl of the culture with approx. $OD_{620\ nm} = 0.10$, was taken and was inoculated in the strip containing sugars.
- After inoculation of the culture in the strips containing sugar, the strips were incubated for 24 to 48 h. The colour change in the sugar strips was noticed which confirmed the fermentation by different bacterial isolates.
- For catalase test, a 50 µl of 3.0% hydrogen peroxide was added in the well and the effervescences were observed for a positive catalase test.

A. HiLacto Identification Kit **B. Strips containing sugars**

Figure 2.2: HiLacto Identification Kit for the assessment of fermentation of different sugars by bacterial isolates

2.2.3 Safety attributes

Haemolytic activity

The safety attributes of the isolates was tested by determining the hemolytic activity of isolates on blood agar plates (Himedia Laboratories, Mumbai, India) (5% sheep blood) [14].

- The blood agar plates were streaked with bacterial cultures (overnight grown) and incubated overnight at 37°C.
- The plates were observed for different types of hemolytic reactions i.e. α, β or ψ hemolysis after incubation.

Antibiotic susceptibility test

The antibiotic susceptibility of different isolates was determined by using the disc diffusion method. The experiment was done by following the recommendations/ guidelines of National Committee for Clinical Laboratory Standards [15].

Overnight grown cultures of bacterial isolates with OD_{600nm} = 0.5 were used for test

A 100 µl of culture broth was spread on Muller Hinton Agar plates with the help of glass spreader

The plates were allowed to absorb the inoculum and the different antibiotic discs were placed over the inoculated plates

The plates were incubated for 24 h at 37°C and were examined for the zone of inhibition surrounding the antibiotic discs

The results were recorded on the basis of diameter of zone around the disc as sensitive (S) and resistant (R), as per European Food Safety Authority (EFSA) 2008

2.2.4 PCR identification of isolates

Bacterial DNA isolation

The extraction of the genomic DNA from bacterial cultures was performed using phenol chloroform protocol

All the isolated bacterial cultures were grown in MRS broth for overnight incubation

The cells were harvested by centrifugation the cultures at for 10 min and the pellet was mixed with the 1 ml of extraction buffer (1 M Tris-HCl (pH 8.0), 5 M NaCl and 0.5 M EDTA)

The mixture was kept at 70 °C for 30 to 45 min following gentle shaking every 5 min and centrifuged at 10,000 rpm for 10 min

The supernatant after centrifugation was collected and to this phenol-chloroform (1:1) was added and vortexed

The suspension was centrifuged at 10,000 rpm for 3 min for separation of organic and aqueous layers which was collected in a fresh centrifuge tube to which ice cold Isopropanol (1:1).

The tubes were kept at -80°C to for 30 mins to precipitate DNA and centrifuged at 10,000 rpm for 5 min

The obtained DNA pellet was washed twice with ice cold ethanol and The DNA pellet was air dried for 15 min and then resuspended into 100 µL nuclease free sterile water

The isolated DNA was visualized in 0.8% agarose gel containing ethidium bromide (0.005%) using UV Transilluminator

16S rRNA gene sequencing

The isolates were identified targeting 16S rRNA gene region using the following PCR components and primer sets [16].

The PCR reaction was carried with total volume of 25µl containing following:

Ingredient	Amount
10× PCR buffer	2.5 µl
25 mM $MgCl_2$	0.5 µl
10 mM dNTP (Promega) mix	0.5 µl
10 pmol, LAB specific primers	1 µl each
5 U Taq DNA polymerase (Intron)	0.25 µl
DNA template	1 µl
Nuclease free Water	19.25 µl

"Primer sequence: SGLAB0159f: 5'GGA AAC AGR TGC TAA TAC CG3' and SGLAB0667r: 5'CAC CGC TAC ACA TGG AG3') and Bact27f (5′-GTTTGATCCTGGCTCAG-3′) and 1492r (5′- CGG CTA CCT TGT TAC GAC-3′) as universal primers" was used to amplify LAB specific genes.

- The PCR conditions used for amplification of target DNA were:
  - Initial denaturation step : 7 minutes at 95°C, amplification of 35 cycles
  - Denaturation step : 1 minute at 94°C.
  - Annealing : 1 minute at 56°C (for LAB specific primers)/ 40 sec at 52°C (for universal bacterial primers).
  - Final extension : 10 min at 72°C.
- The PCR amplified DNA fragments were visualized in a UV trans-illuminator.
- The PCR product was sequenced from Eurofins Genomic India Pvt Ltd.
- The obtained sequences of amplified product was matched to the sequences from the GenBank database using BLAST (Basic Local Alignment Sequence Tool) to identify the microbial genera.

- The sequences were subjected to multiple alignment using by using Clustal Omega.
- The phylogenetic trees were constructed by the neighbour-joining method.
- The identified target sequences were submitted to NCBI gene bank database and used throughout the study.

2.3 Results and discussion

2.3.1 Isolation and identification of the isolates

Out of 150 samples, 101 isolates were obtained on MRS agar plates having opaque, white and creamish colonies which were further identified as Gram positive rods and cocci, facultative anaerobes and catalase negative. These isolates were further shortliested based on their safety attributes depicting hemolytic activity on sheep blood agar plates. Out of 101, 51 isolates were found to be non-hemolytic which were further screened for the passage of GI tract conditions (Table 2.1). Out of 51 isolates, 15 isolates were able to pass through the acidic pH of stomach and bile salt conditions of intestine. These 15 isolates were further investigated for simulated GI tract conditions and further checked for *in vitro* and *in vivo* probiotic attributes as per joint protocols given by "DBT-ICMR, Govt of India" (Fig. 2.3).

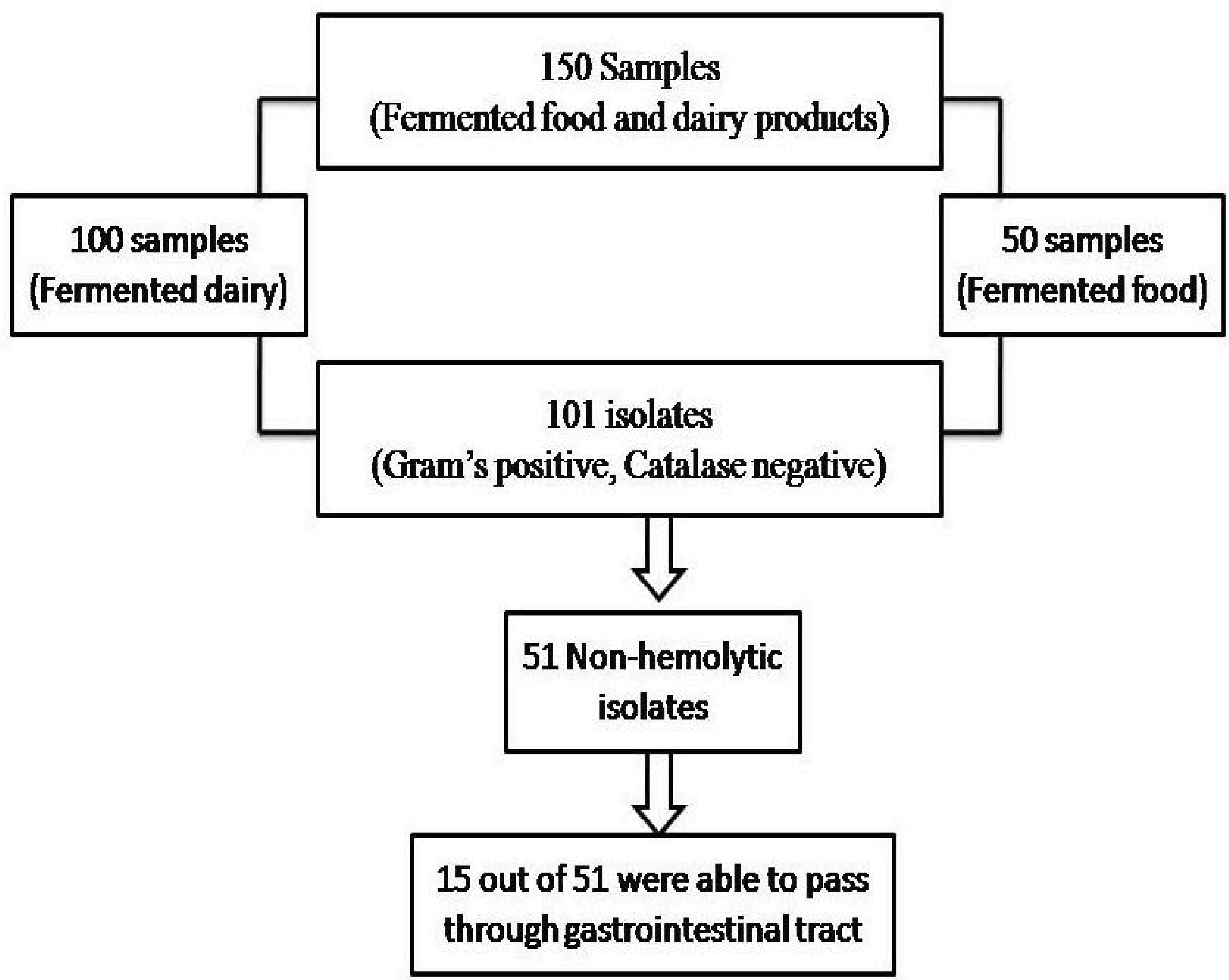


Figure 2.3: Subtractive screening of isolates

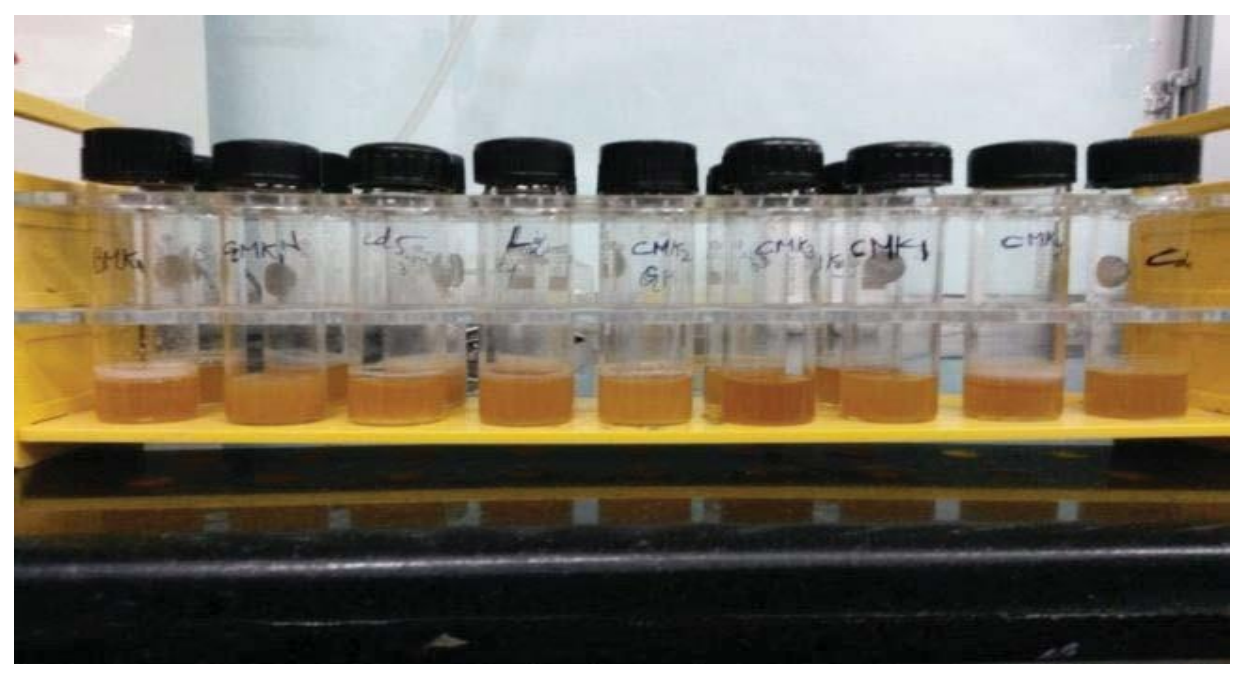


Figure 2.4: Enrichment of pure colonies of isolates in MRS broth

Table 2.1: Details of sampling for isolation of LAB from different fermented dairy products

Origin of Samples	Samples collected	Distinct colonies on MRS agar	Isolates without hemolytic activity	Isolates surviving simulated gut conditions
Cow milk	20	16	9	1
Buffalo milk	20	12	5	1
Goat milk	20	10	4	1
Curd	20	18	13	3
Buttermilk	20	10	7	2
Fermented dough	50	26	13	7
Total number	**150**	**101**	**51**	**15**

2.3.2 Biochemical activities of bacterial isolates

The biochemical activities of isolated bacterial cultures (Fig. 2.4) for the utilization of different sugars are presented in Table 2.2. All the 15 cultures indicated distinctive reactions for use of various sugars. The color change was noticed after incubation of each well with different cultures. The observations were recorded as good, doubtful or inconclusive in terms of change in color. The observed reactions were compared as per the standards provided by manufacturer. Variable outcomes were obtained for different sugars for different isolates. Among the tested sugars, raffinose, arabinose, mellibiose and trehalose were not metabolizable by the isolates. The galactose, maltose and mannose were fermented by most of the isolates. The different metabolic activities of the cultures to different sugars could be attributed to presence of different hydrolases and sugar transport systems in each bacterial species investigated in the present study. Moreover, the carbohydrate fermentation assays don't completely uncover the genuine character of the strains. The different sugar fermentation capabilities have been reported to vary among different starters.

2.3.3 Antibiotic susceptibility pattern

The strains were further evaluated for their antibiotic susceptibility patterns for their safety viewpoints as these bacterial cultures may express some pathogenic attributes and sensitivity towards routinely used antibiotics (Fig. 2.5) i.e. LAB may express certain virulence genes and/or antibiotic resistance genes. The antibiotic susceptibility pattern of bacterial isolates is presented in (Table 2.3). Among all the tested strains, the maximum resistance was observed for the isolate HM29 from milk sample which highly resistant to all the antibiotics used in the present study. Majority of the other isolates were observed to be resistant to vancomycin, kenamycin, penicillin, bacitracin, and clindamycin and susceptible to amoxyclav and carbemicillin. The resistance to vancomycin was also observed for majority of the isolates. A review survey on pathogenic significance of Lactobacillus-related contaminations revealed that the lactic isolates were additionally generally susceptible to antibiotics, with the exception of the inborn protection from vancomycin which remained similar for vulnerability towards erythromycin and resistant to

vancomycin. The intrinsic resistance to vancomycin has been reported in most of the lactic cultures such as *L. plantarum, L. paracasei* and *L. salivarius*. The results pertaining to differences in these ntibiotic susceptibilities in this investigation needs further examination to decide whether the resistance is intrinsic or transferable. The outcome of this resistance pattern will be helpful in further evaluation of wellbeing of these bacterial isolates for human utilization through established criteria, guidelines.

Table 2.2: Carbohydrate fermentation tests for identification of different bacterial isolates from food products

Isolates	Esculin	Catalase	Xylose	Cellobiose	Arabinose	Maltose	Galactose	Mannose	Melibiose	Raffinose	Sucrose	Trehalose
CD4	P	N	W	N	N	P	P	W	P	P	P	W
BTM7	P	N	W	P	W	P	P	P	P	P	P	P
BTM9	P	N	W	W	P	P	W	N	N	W	W	N
CD13	N	N	W	P	N	P	W	P	W	P	W	W
GTM14	P	N	P	W	N	P	W	P	N	N	N	N
HM29	N	N	P	W	N	P	P	W	P	W	P	W
HM34	P	N	P	P	N	P	N	P	W	N	W	P
CD1	P	N	P	P	P	W	W	P	P	P	W	P
K75	N	N	N	N	N	P	W	W	N	N	P	N
K78	N	N	P	N	N	P	W	N	N	P	P	W
K84	P	N	W	P	P	P	P	P	W	N	P	P
K90	P	N	W	P	W	P	P	P	P	N	P	P
K94	P	N	P	P	P	P	P	P	N	N	N	N
K98	N	N	P	P	P	P	P	P	N	N	N	N
K100	N	N	P	N	N	P	W	N	P	P	P	N
L. rhamnosus GG	P	N	P	P	W	W	P	P	P	P	W	N

P: Positive (pH<5.2), N: Negative (pH 5.2 to 6.8), W: Weak (pH 5.2)

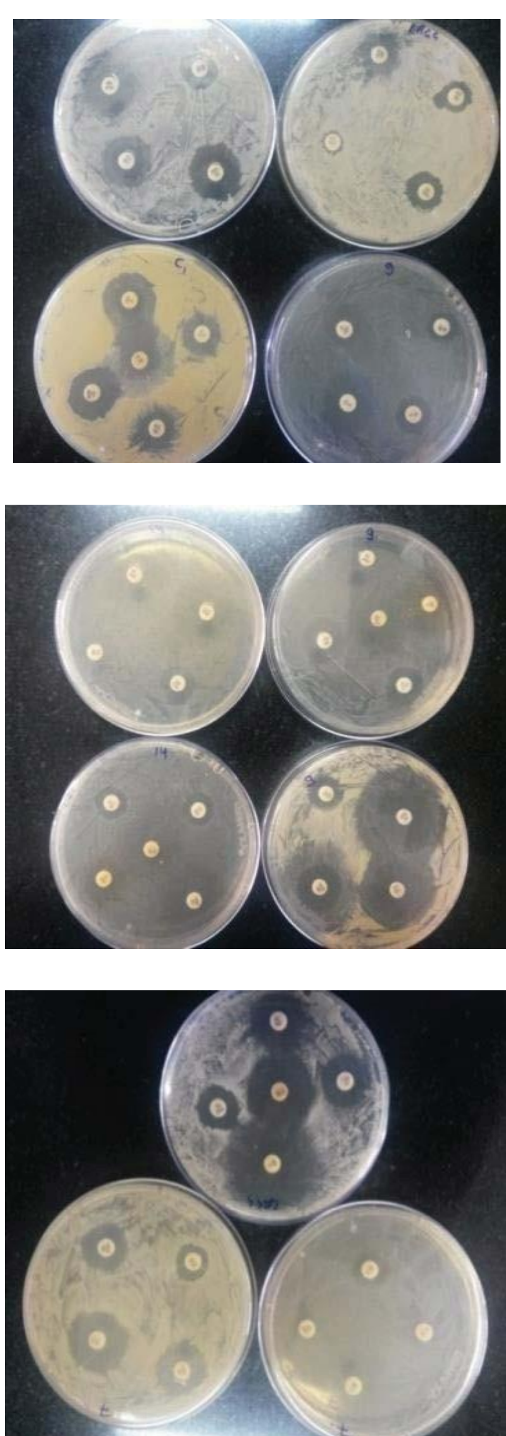


Figure 2.5: Antibiotic susceptibilites and resistance of bacterial isolates against various antibiotics

Table 2.3: Antibiotic susceptibility pattern of bacterial isolates obtained from different milk and cereal based food products

Isolates	Amp	Ak	Amc	Cb	Rif	Cd	K	B	E	Hlg	Cep	Tob	Va	P	Te
CD4	R	R	R	S	R	R	R	S	R	R	S	R	R	S	R
BTM7	S	S	S	S	S	S	R	R	S	R	R	S	S	R	S
BTM9	S	S	S	S	S	S	S	R	S	S	R	R	R	R	S
CD13	R	S	S	R	R	R	R	R	S	S	S	S	R	R	R
GTM14	S	R	S	S	S	R	S	R	S	R	R	R	R	R	S
HM29	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
HM34	R	S	S	S	S	R	R	R	S	S	S	R	R	R	R
CD1	R	R	S	S	S	R	R	R	S	S	S	S	S	R	S
K75	S	S	S	S	S	S	S	R	S	R	R	R	R	S	S
K78	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K84	S		R	S	R	R	R	R	S	R	S	S	S	S	R
K90	S	S	S	R	R	R	S	R	S	R	R	R	R	S	S
K94	S	R	S	S	S	R	R	R	S	R	S	S	R	S	S
K98	S	R	S	S	S	R	R	R	R	R	S	R	R	S	R
K100	S	R	S	S	R	R	S	R	R	R	R	R	S	R	R
L. rhamnosus GG	R	R	R	S	R	R	R	S	R	R	S	R	R	R	R

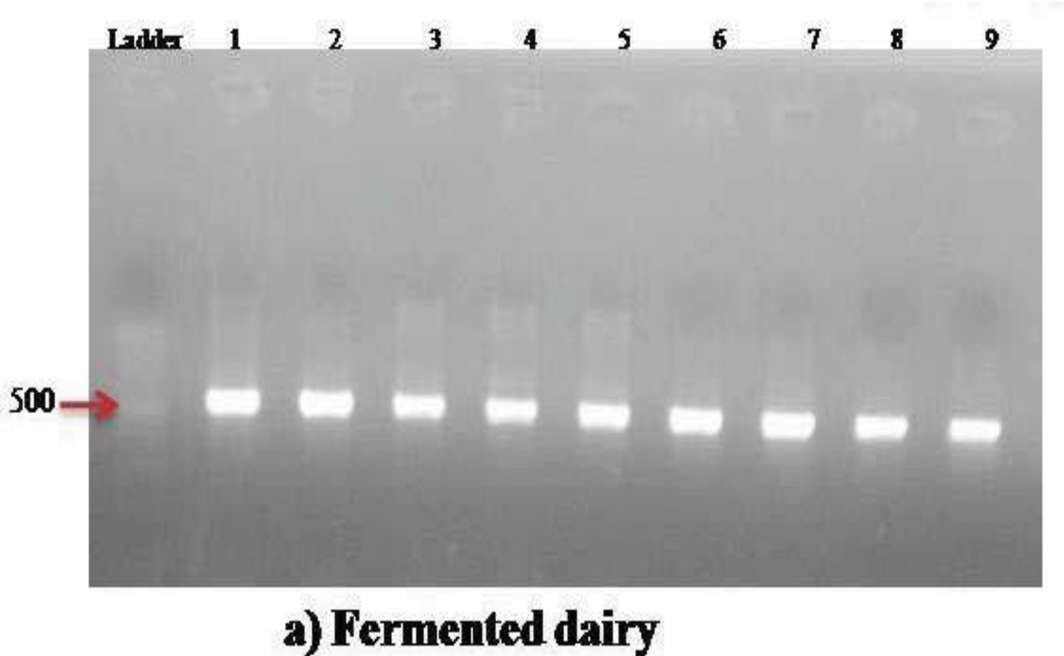
"Antibiotic Susceptibility: S=Susceptible, R=Resistant: AMP – Ampicillin (10 mcg), Amc – Amoxyclav (30 mcg), CB – Carbemicillin (100 mcg), RIF - Rifampicin (5 mcg), CD-Clindamycin(2 Mcg), K-Kenamycin (30mcg), E-Erythromycin (15U), Va -Vancomycin (30 mcg), P-Penicillin (10 U), TE-Tetracycline (30 Mcg), HLG – Gentamycin (120 Mcg), AK (Amikacin), B(Bacitracin), CEP (Cephalothin), TOB (Tobramycin)"

2.3.4 Molecular Identification of Isolates

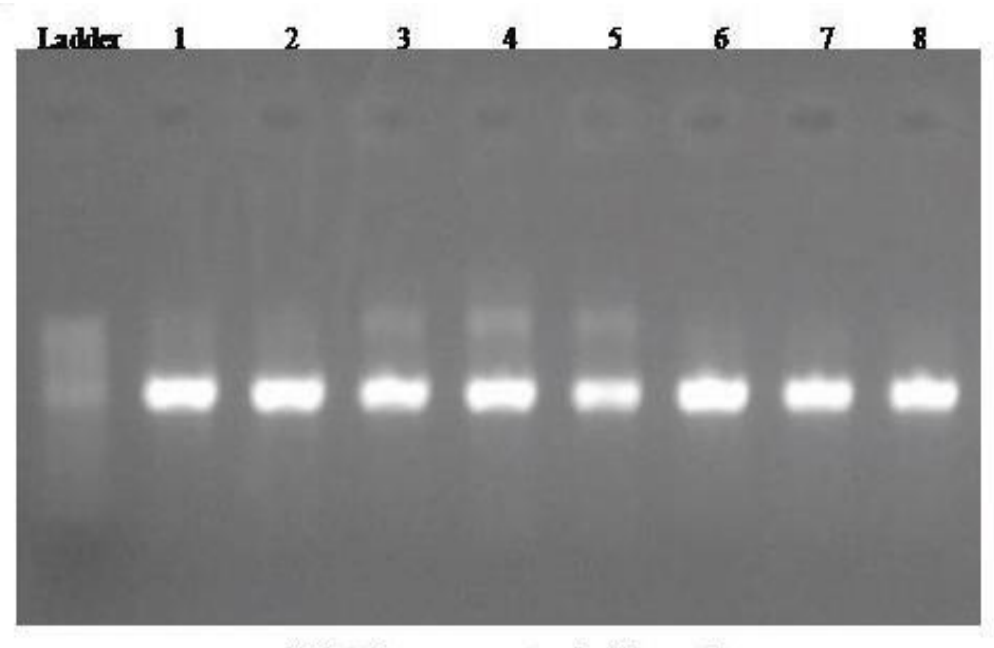
The most promising isolates were identified using 16S rRNA gene sequencing and the list of identified isolates is given in Table 2.4. Most of isolates were identified from group Lactic acid bacteria and were further investigated for probiotic *in vitro* and *in vivo* attributes.

Table 2.4: Accession numbers of isolates identified by 16S rRNA gene sequencing

Isolates	Identified isolates	Accession number
CD4	*L. paracasei* CD4	KX398050.1
BTM7	*L. gastricus* BTM7	KX398051.1
BTM9	*Brev. aydinogluensis* BTM9	KX583600.1
CD13	*Brev. thermoruber* CD13	KX583601.1
GTM14	*Enterococcus sp* GTM14	KX583602.1
HM29	*Brevi. thermoruber* HM29	KX583603.1
HM34	*Brev. thermoruber* HM34	KX583604.1
CD1	*W. confuse* CD1	KX583605.1
K75	*L. fermentum* K75	MF927597.1
K78	*L. fermentum* K78	MF927688.1
K84	*L. plantarum* K84	MF455228.1
K90	*L. plantarum* K90	MF455229.1
K94	*P. acidilactici* K94	MF455252.1
K98	*P. acidilactici* K98	MF457590.1
K100	*L. fermentum* K100	MF944224.1



1, CD4; 2, BTM7; 3, BTM9; 4, CD13; 5, GTM14; 6, HM29; 7, HM34; 8, CD1; 9, LRGG



1, K75; 2, K78; 3, K84; 4, K90; 5, K94; 6, K98; 7, K100, 8, LRGG

Figure 2.6: Figures showing the amplified PCR products on agarose gel

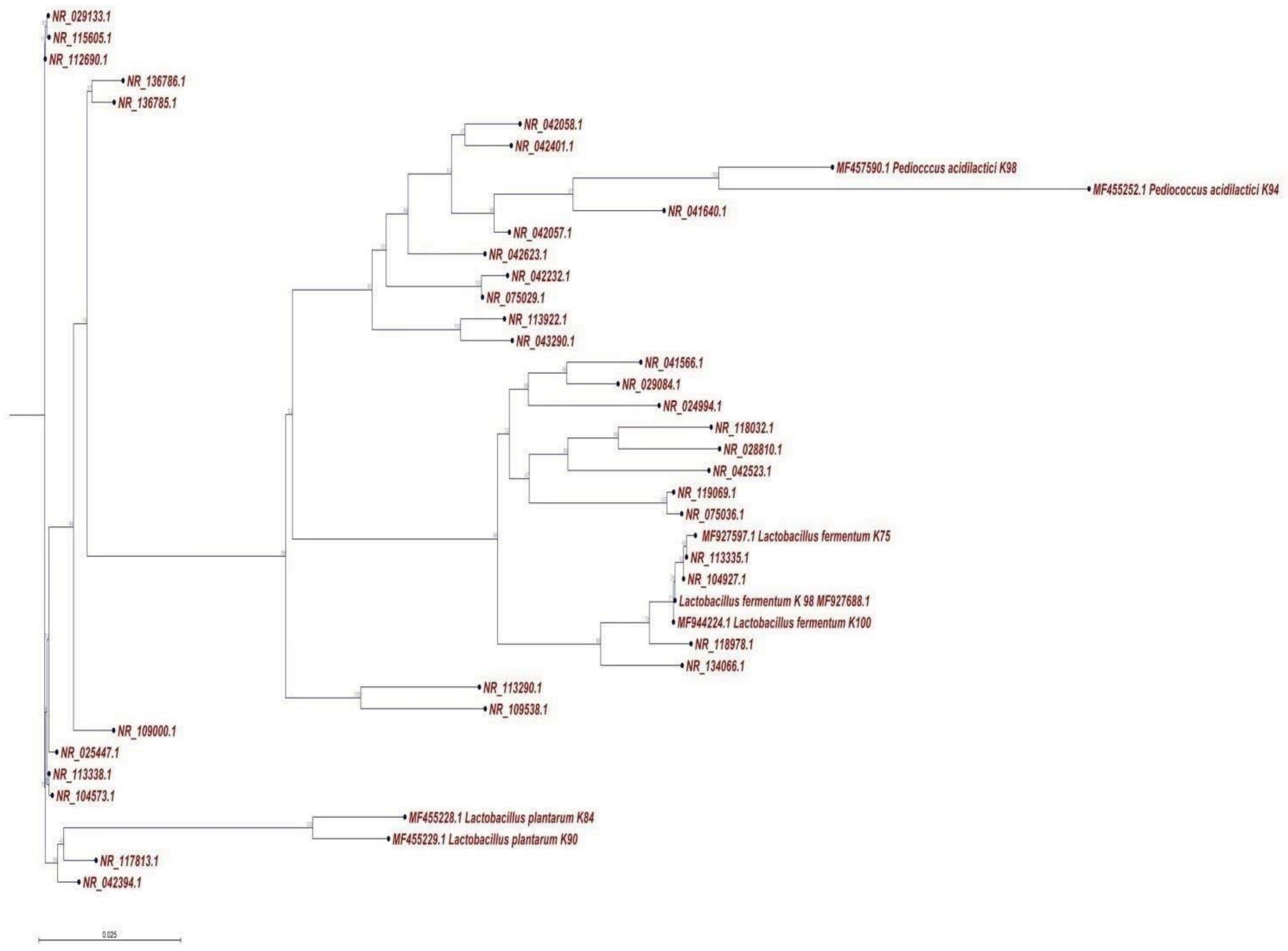


Figure 2.7: Phylogenetic analysis of the isolates using BLAST

2.4 Conclusions

The results obtained in this study indicated that dairy and cereal based fermented foods are reservoir of beneficial bacterial cultures mostly belonging to the group of lactic acid bacteria. Most of the isolates were obtained from fermented dairy of cereal products as those were fermented with diverse group of microorganisms. From a total of 51 non-hemolytic isolates, twenty were obtained from fermented foods indicating that higher prevalence of hemolytic cultures in non-fermented food products.

Chapter 3

To screen the bacterial isolates for probiotic attributes using *in vitro* methods

3.1 Introduction

Lactic acid bacteria (LAB), group is extensively used in the production of fermented food for preservation as well as fermentation. Few members of this group are well recognized as probiotics, which are nonpathogenic microorganisms [1] include different bacterial genera with documented benefits on health, such as species of *Saccharomyces, Lactobacillus, Bifidobacterium, Streptococcus, Pediococcus, Leuconostoc, Enterococcus, Escherichia coli,* and *Bacillus.* These genera are generally regarded as safe (GRAS) [2]. In the process of fermentation LAB are implicated among these genera, and among all the dairy fermented products it acts as most dominant microflora. Their role in food preservation is well known; they inhibit the expansion of spoilage microorganisms or pathogens borne through food by producing antimicrobial substances [3]. LAB probiotic consumption has been reported to control some infections (diarrhea and urogenital infections), enhances the resistivity towards malignancy, modulates the immune system [4], slows down the development of cancer (colon), decreases the amount of cholesterol, mitigates the level of constipation, and provides prevention from allergies through food. Furthermore, for the better immune system they contribute to the prevention of antibiotic-associated diarrhea [5].

LAB are used as probiotic starters and natural micro flora of dairy products; their presence enhances the nutritive value of fermented foods, therefore, the fermented food products are considered as the most appropriate source for probiotic delivery. Despite the associated health benefits, many fermented cereal products have not been adequately explored from the viewpoint of probiotic lactic starters. Consequently, new ecological niches are being subjected to bioprospecting for novel probiotics. Different fermented cereal products provide wide range of health advantages depending on the probiotic strains of LAB they contain [6]. Throughout fermentation of these cereal fermented products, the microbial enzymes act on the substrate and produce substantial changes in the organoleptic, nutritive, physic-chemical structure o the food product. The fermentation also alters the contents of bioactive molecules whereby enabling the fermented food as more beneficial than non-fermented food for human consumption [7]. Cereal

fermentation by LAB increases soluble fibers accessibility, phenolic resin acids, and also the bioactive peptides by degradation of proteins present in the fluor [8]. The organoleptic and technological characteristics of flour are improved by fermentation. Furthermore, it improves the crumb structure loaf volume, and shelf life of bread.

Fermentation is a process involved in the preparation of various traditional delicacies in villages in Himachal Pradesh, a state in North Western India. Traditional food processing technologies use available raw material and are subject to socio-cultural ethos, ethnic preference, and religion [9]. Many rural districts in Himachal Pradesh consume an indigenous bread or *roti,* known as *babroo*, as part of their staple diet. Naturally fermented flour dough of wheat is used to prepare *Babroo* [10]. A mixture of wheat flour, water, and some sugar is kneaded until a dough-like consistency is observed. The dough is incubated over \night at room temperature and fermented naturally. The fermented dough is rolled into *roti* and cooked bread for consumption. It has been reported from the previous studies that the fermentation of *babroo* dough is brought about by microflora, such as *Debaromyces sp.*, *Saccharomyces cerevisiae*, *Lactococcus lactis,* and *L. plantarum* [11]. However, the functional and probiotic activities of the isolates from fermented wheat dough or dairy products from Himachal province have not been reported up until now. Therefore, the objective of this study was to determine the functional as well probiotic activities of different cultures obtained from a wide range of food products as reported in Chapter 2 using *in vitro* methods.

3.2 Methodology

3.2.1 Screening of bacterial cultures for probiotic attributes

During the harsh conditions of gastrointestinal tract, the bacterial culture survival was assessed by simulating the conditions of the gastrointestinal tract *in vitro* and culturing the bacterial isolates in these conditions. The survival was determined by observing the viable cell count of specific strain under those conditions at different time intervals.

3.2.2 Survival under simulated gastric and intestinal conditions

- For simulated gastric juice, pepsin (3 mg/mL) was added to sterile solution of saline (0.85% NaCl w/v).
- The pH of the fluid was adjusted to 2.5 using 1N HCl.
- The simulated gastric fluid was inoculated with overnight grown bacterial cultures (~7.70 log CFU/ml) and incubated for 2 hours.
- The pancreatic juice contained pancreatin (1 mg/mL) and 0.5 % of Ox-gall Bile in sterile saline (0.85% NaCl, w/v).
- The pH of pancreatic fluid was adjusted to 8.0 by 1M NaOH. The prepared solution was used as simulated intestinal fluid.
- The survival of bacterial cultures in this intestinal fluid was determined for a duration of 6 h.
- In order to determine the survival to gastric and intestinal fluids, viable count the cultures were determined on MRS agar at regular intervals of one hour [12].

3.2.3 Adhesion potential of bacterial cultures

Cellular auto aggregation

The ability of cells for auto aggregation was determined as per the protocol of [13].

All the isolated bacterial cultures were grown in MRS broth for overnight incubation

After incubation, the cells were harvested by centrifugation the cultures at for 10 min

The cell pellet obtained was washed twice with phosphate buffer saline (PBS) and was resuspended in PBS

The absorbance of cell pellet in PBS was set to 0.7 as the initial absorbance () at 600 nm

The suspension process of bacteria was centrifuged and also pellet was again suspended in an associate same volume of MRS broth

The suspension process of bacteria was centrifuged and also pellet was again suspended in an associate same volume of MRS broth

For a reference the final absorbance which is () of the suspension layer at upper level was measured by making use of uninoculated MRS broth.

The difference in the absorbance (in percentage) was calculated and presented as cellular autoaggregation index

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➢

The formula for calculating the percentage of aggregation is as follows:

$$Aggregation\ (\%) = 100 \times \frac{(Abs_{Initial} - Abs_{Final})}{Abs_{Initial}}$$

Cell surface hydrophobicity of the bacterial isolates

This property of isolates was assessed by exploiting the protocol of [14].

All the isolated bacterial cultures were grown in MRS broth for overnight incubation

After incubation, the cells were harvested by centrifugation the cultures at for 10 min

The cell pellet obtained was suspended in Phosphate urea magnesium (PUM) buffer having a pH of 7.0. The cell biomass was washed twice in PUM buffer

The pellet was resuspended in the same buffer and the initial value of absorbance at 600 nm () was fixed to 0.7 OD

The cell suspension in PUM was mixed with n-hexadecane in a ratio of (3:1), vortexed, and incubated at 37°C for around 10 min

The mixture was vortexed again, and phase separation was allowed by further incubation of the suspension at 37°C for around 1 h

After phase separation, the aqueous phase was aspirated gently and the absorbance the aqueous phase was determined at 600 nm

The difference in the absorbance (in percentage) was calculated and presented as cell surface hydrophobicity

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- The percentage of surface hydrophobicity was calculated using the following formula:
-

$$Surface\ Hydrophobicity\ (\%) = 100 \times \frac{(Abs_{Initial} - Abs_{Final})}{Abs_{Initial}}$$

Adhesion of the bacterial isolates to Caco 2 cell

- The Caco 2 (ATCC HTB 37) was procured from National Center of Cell Science, Pune (Maharshtra, India).
- The procured Caco 2 cells were cultured in Dulbecco's modified Eagle's Minimal Essential Medium (Sigma Aldrich) supplemented with fetal bovine serum with 20 percent (v/v) and (100IU/mL and 100 μ g/mL) of Penicillin–Streptomycin
- The cultured cells were incubated in a humidified atmosphere containing 5 % of CO_2 at the temperature of 37°C.
- The bacterial cultures grown in MRS broth were centrifuged and washed with ringer solution.
- The cell pellet obtained was suspended in DMEM medium having pH value of 4.5.
- A 12-well tissue culture plate was used for performing the microbial adhesion test (Thermo Fisher scientific, USA).
- With the density of 2×10^5 cells per well, the Caco 2 cells were seeded. For every well that contains a monolayer of Caco 2 was thereby added with the bacterial suspension of around 1mL ($0.5 \times TT^5$ CFU/mL).
- The bacterial cells were allowed to adhere to Caco2 cell by incubating the suspension for around 1 h at the temperature of 37°C.
- After incubation, the contents in the well were washed with phosphate buffer saline (PBS), twice in order to remove the unbound microorganisms from the wells.
- The bacteria adhered to the Caco2 cells were thereby fixed by making use of p-formaldehyde 1% (w/v) and further stained by Giemsa stain.
- The preparation was observed in light microscope with the magnification of 20 X [15].

Adhesion capacity of the probiotic isolates to intestinal mucus

The Giemsa staining approach was used to study the adhesion potential of bacterial cultures to mucin from pig's intestine [16].

- The mucin adhesion assay was performed on microtiter plates where first mucin was coated on the plates followed by addition of bacterial suspension.
- A 150 µ L of porcine mucin (Sigma-Aldrich, GmbH) was allowed to get coated over the wells of micro titer polystyrene plates by incubating the plates overnight.
- After coating of mucin, 100 µ L of each overnight grown bacterial culture was added to the wells.
- The cultures were allowed to adhere to the mucin by incubating the plates at 37°C for one hour.
- After incubation, the non-adherent cells from the plates were removed by washing thrice with 250 µL of phosphate buffer saline.
- The adherent cells were fixed at the temperature of 60°C and the cells were stained for the duration of 45 min using crystal violet (100 µL/well, 0.1% solution)
- After staining, the excess of the stain was removed, and the wells were washed with PBS.
- The stain from the adherent cells was solubilized using 100 µ L citrate buffer (pH 4.3) by adding the buffer and incubating the plates for duration of 45 min.
- The absorbance of the stain was measured using microplate reader (Multiskan GO, Thermo Scientific, USA).
- The results were compared with the control taken as stained mucus with no addition of bacterial culture.

3.2.4 Screening of probiotic LAB for functional properties

Antioxidant activity

The ability of the isolates to scavenge free radicals was determined by two well established methods using DPPH and ABTS as substrate for generation of free radicals

DPPH assay

- In order to detect the ability of bacterial cultures to scavange free radicals, the filter sterilized cell free extract of bacterial cultures was used [17].
- A 500 µL of Cell free extract was added in the test tubes followed by addition of freshly prepared 3.0 ml DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate, 0.005 percent in ethanol) solution.
- The tubes were incubated in the dark for a duration of 30 min.
- After incubation, the absorbance of DPPH was determined at 517 nm taking uninoculated MRS broth as blank.
- For comparison 100 µg/mL of pure ascorbic acid was taken as natural standard.
- All the experiments were done in triplicates and the values were represented as mean values.
- The % free radical scavenging activity was calculated as:

$$Free\ radical\ scavenging\ activity\ (\%) = \frac{As_{517\ control} - As_{517\ sample}}{As_{517\ control}} \times 100$$

ABTS assay

- A solution of ABTS was obtained by mixing stock solution of 7mM (2, 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) with 2.45 mM of potassium per sulfate (1:1, v/v).
- The ABTS solution prepared was stored for the time till the process of reaction and stability in absorbance is completed.
- The OD of ABTS solution was adjusted to 0.700 at 734 nm using ethanol. The freshly prepared ABTS solution was used for the study.

- For estimation of free radical scavenging activity, 3mL of ABTS was added to 500 µL of cell free extract from each culture.
- The tubes were incubated in the dark for 30 min.
- After incubation, the absorbance was taken at 734 nm using microplate reader.
- Ascorbic acid (100 µg/mL) was taken as natural standard.
- All the experiments were done in triplicates and the values were represented as mean values.
- The calculation of percent free radical scavenging is formulated as:

$$Free\ radical\ scavenging\ activity\ (\%) = \frac{As_{734\ control} - As_{734\ sample}}{As_{734\ control}} \times 100$$

Bile salt hydrolase activity

- The bacterial isolates were grown in MRS broth and activated culture was streaked on MRS agar plates containing 0.037 %t of $CaCl_2$ and 0.5% of Sodium taurodeauxycholic acid.
- The inoculated plates were incubated for 72 h at 37°C. during the incubation the plates were observed for the presence of hydrolysed products of salts that precipitated in agar medium in or around the bacterial colonies
- The plates indicating precipitation were considered to exhibit bile salt hydrolase activity [18].
- The bile salt hydrolase activity was confirmed by confirming the bsh activity by amplification of bsh gene
- Species specific primers was used for detecting the presence of *bsh* "(Primer sequence: bsh-f: 5′GGTTGGTCGGCCAGTTCTTT3′ and bsh-r: 5′CCA ACA TGC CCA AGT TCG AC3′)" (Turpin *et al.*, 2011).

- The amplification of extracted DNA was done at specified conditions as described by Turpin *et al.*, 2011
- The PCR product was visualized using UV-transilluminator.

Cholesterol-lowering assay

The cholesterol lowering potential of bacterial isolates was determined using spectrophotometric method.

All bacterial cultures were grown in MRS broth supplemented with with 100 µg/ml of filter sterilized cholesterol (and 0.3 percent (w/v) Oxgall bile

The inoculated tubes were incubated at 37C for 24 h and centrifuged at for 10 min and the supernatant was collected in the fresh tube

1 mL of supematant, 1 mL of Potassium Hydroxide (33% w/v) and absolute ethanol (2 mL) were mixed for the duration of 1 min and heated at 70°C for 30 min

After cooling, hexane (5 mL) was added, vortexed for 1 min and the tubes were left at room temperature for 30 min.

After phase separation, the hexane layer wss transferred in a clean glass tube and the hexane was evaporated

To the residue 2mL of o-phthalaldehyde followed ollowed by addition of 1 mL of sulfuric acid. The contents were mixed

The tubes were vortexed for one min and the absorbance of the contents was measured at 540 nm

$$\text{Cholesterol lowering activity } (\%) = \frac{As_{540\ control} - As_{540\ sample}}{As_{540\ control}} \times 100$$

Exopolysaccharide (EPS) production

- The capability of the isolates towards the production of exopolysaccharide (EPS) was assessed on the MRS plates which was supplemented with 1% sucrose.
- The overnight grown bacterial cultures were streaked on the agar plates and further incubated at the temperature of 37°C for the duration of 72 h.
- After incubation, the growth pattern of bacterial cultures was observed. The presence of mucoid surfaces around the colonies was taken as positive response towards production of EPS.

3.3 Results

3.3.1 Screening of bacterial cultures for probiotic attributes

Tolerance to gastrointestinal tract conditions

Probiotic cultures should necessarily be resistant to bile salts and highly acidic conditions to survive in the GI tract which is the first criteria to be followed during selection of a probiotic candidate. As per the mutual protocols provided by" DBT-ICMR" the evaluation of bacterial isolates was done for their probiotic characteristics. The first basis standard for the evaluation of potential probiotic strains is that, it must be capable enough to bear the environmental conditions of human GI tract, such as acidic condition, resistance to pancreatin, bile, and pepsin. The survival of isolates to these *in vitro* simulated conditions is presented in Table 3.1. All the isolates, except for *Brevibacillus aydinogluensis* BTM9 and *B. thermoruber* HM29, exhibited growth in the pH range 2.0–7.0. The isolates were tolerant to bile and pancreatin at concentrations of 1% and 1mg/mL, respectively. All the isolates were able to withstand the harsh conditions in intestine by surviving in bile salt conditions and possessed bile salt hydrolase activity (Results in the following sections).

Highly acidic or low-pH environments have been reported to reduce the metabolism and activity of bacterial cultures during their exposure to low pH of gastric juice (pH $\leq$ 2.0) over a period of time. We have determined the survival of bacterial cultures at pH 2.0 anD 3.0 as these two pH conditions are reported as a threshold to determine acid resistance in probiotic cultures because these conditions simulate the environment encountered by the bacteria in the stomach [19]. The ability of the isolates to simulated gut conditions at low pH and in the presence of bile salts

revealed that the CFU count of the bacteria decreased by nearly one log unit in the CFU count after 1h of incubation at acidic pH of 2.0 ($p<0.05$); however, an further increase in pH to 3.0, this decrease in count was not significant. The results indicated that these strains are acid tolerant with higher adaption to higher pH used in the MRS broth. In the conditions that simulate the small intestinal (pH 8.0) conditions, no decrease in bacterial counts was observed even after 6 h of incubation indicating that all the tested bacterial strains exhibited acceptable survival in intestinal fluid ($p>0.05$). For the conditions of low pH and bile salt, most of the isolates exhibit acceptable tolerance. However, the rate of survival for of bacterial strains to these conditions was strain specific. It has been reported from various research studies that the lactic culture potential to survive under these conditions is dissimilar when these are isolated from different fermented foods. The probiotic prospective of LAB cultures has been previously reported which were isolated from the conventional fermented foods in Ladakh region [20]. Similar results were reported with LAB cultures that are isolated from Kimchi, Korean conventional fermented vegetable [21], some other fermented foods of Korea [22], kefir [23]. In these studies, the isolated cultures met the criteria of potential probiotics by exhibiting tolerance to gastrointestinal conditions.

From our results, it is evident that the isolated LAB exhibited higher survival rates in the pH range of 2.0-3.0 and in presence of pancreatin up to a concentration of 1mg/mL of, however, a slight decline was observed at pH 2.0. The *L. gastricus* strain BTM7 and *L. paracasei* strain CD4 were identified as potential candidates as these isolates were able to grow at pH 2 even after the incubation of 4 h.

In addition to low-pH tolerance, the tolerance of LAB towards bile salts is equally important because bile salts exhibit antimicrobial properties. Therefore, in order to ensure the colonization of human intestine and in order to maintain the metabolic activities in bacteria, the tolerance to bile salts is prerequisite [24]. The recommended range of bile concentrations for selecting probiotic bacteria for human use is 0.15 percent to 0.3 percent [25]. However, in small intestine the average concentration of bile salts is approximately in a range of 0.2 percent to 2 percent (w/v), which is subjected to factors like diet ingested [26]. Therefore, in this study, the tolerance of all the LAB isolates was tested up to a concentration level of 1 percent. It was observed that at

a bile salt concentration of 1 percent, all the lactic cultures were capable to survive with no significant effect on viable cell count. The appearance of white precipitated zone around the colonies also indicated that the isolates were able to hydrolyze the bile which was also confirmed by molecular analysis targeting bsh gene.

Table 3.1: Tolerance of selected isolates to gastrointestinal tract conditions (low pH and bile)

LAB strains	**Gastric phase (Log CFU/ml)**			**Intestinal phase (Log CFU/ml)**		
	0 h	**1 h**	**2 h**	**1h**	**4 h**	**6 h**
***L. paracasei* CD4**	6.21^{a}	6.08^{bc}	5.99^{c}	7.87^{d}	7.74^{b}	7.47^{ab}
***L. gastricus* BTM7**	6.11^{a}	6.00^{bc}	5.90^{c}	7.79^{bd}	7.63^{b}	7.03^{a}
***Brev. aydinogluensis* BTM9**	6.07^{a}	5.32^{a}	5.12^{a}	7.76^{bd}	7.47^{ab}	7.06^{a}
***Brev. thermoruber* CD13**	6.20^{a}	5.30^{a}	4.77^{a}	7.74^{bd}	7.56^{ab}	7.30^{a}
***Enterococcus sp* GTM14**	6.07^{a}	5.30^{a}	5.04^{a}	7.78^{bd}	7.59^{ab}	7.06^{ab}
***Brevi. thermoruber* HM29**	6.30^{a}	6.04^{bc}	5.77^{bc}	7.65^{bd}	7.62^{ab}	7.53^{ab}
***Brev. thermoruber* HM34**	6.07^{a}	5.60^{ab}	5.30^{ab}	7.84^{cd}	7.80^{b}	7.75^{b}
***W. confuse* CD1**	6.20^{a}	5.95^{bc}	5.87^{bc}	7.56^{ad}	7.40^{ab}	7.42^{ab}
***L. fermentum* K75**	7.49^{b}	6.18^{c}	6.20^{c}	7.28^{ac}	7.32^{ab}	7.40^{ab}
***L. fermentum* K78**	7.58^{b}	6.89^{d}	7.00^{d}	7.26^{ab}	7.26^{ab}	7.32^{ab}
***L. plantarum* K84**	7.41^{b}	6.18^{c}	6.20^{c}	7.00^{a}	7.08^{a}	7.08^{a}
***L. plantarum* K90**	7.68^{b}	7.18^{d}	7.26^{d}	7.06^{a}	7.08^{a}	7.07^{a}
***P. acidilactici* K94**	7.54^{b}	7.00^{d}	7.00^{d}	7.00^{a}	7.32^{ab}	7.32^{b}
***P. acidilactici* K98**	7.32^{b}	6.00^{bc}	6.12^{c}	7.23^{ab}	7.28^{ab}	7.28^{ab}
***L. fermentum* K100**	7.29^{b}	6.02^{bc}	6.22^{c}	7.23^{ab}	7.26^{ab}	7.26^{ab}
L. rhamnosus GG	6.32^{a}	6.14^{bc}	6.04^{c}	7.84^{cd}	7.77^{b}	7.80^{b}

"a-d Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)"

3.3.2 Adherence potential of probiotic isolates

Cell surface hydrophobicity and cellular autoaggregation

The results of cell surface hydrophobicity and autoaggregation for the LAB cultures are shown in Table 3.2. The cellular autoaggregation % in the tested strains ranged from 5-41%. Among each isolates, the *B. thermoruber* CD13 and *L.paracasei* CD4 exhibited the maximum and minimum value of autoaggregation, respectively. The isolates exhibited a hydrophobicity between a range of 15-42% towards n-hexadecane, and *L. gastricus* BTM7 attains the maximum capability for adhering to an organic solvent.

The adhesion and the colonization of intestinal wall by lactic cultures is reported to be affected by the surface properties of probiotic LAB whereas adhesion and colonization are both desirable properties of probiotic LAB. The adhesion capability of the culture is determined targeting surface properties like hydrophobicity and autoaggregation towards organic solvents. Adherence to intestinal walls is a crucial criterion for the establishment of a probiotic; therefore, most promising potential probiotic isolates were subjected to screening for adherence by using organic solvents. The isolates hydrophobicity for n-hexadecane was obtained in the range from 15 to 42 percent. The isolate *L. plantarum* K84 exhibits the maximum hydrophobicity, among other isolates when compared with *L. rhamnosus* GG which is similar to the hydrophobicity by the standard strain ($p>0.05$) On the other hand *Enterococcus* GTM14 exhibits the lowest hydrophobicity towards n-hexadecane, which clearly specifies that for the adhesion point of view the *L. plantarum* K84 is a prospective probiotic culture (Table 3.2). Surface hydrophobicity may vary with cell surface protein expression levels as reported earlier for the probiotic strains [27]. The percent autoaggregation was found to be maximumin *P.acidilactici* K98 (41 %). It has been described in various studies that there is a strong correlation among hydrophobicity and adhesion ability [28][29]. Even though this relation was notices in the present study, still there are few numbers of studies that have reported contradictory correlation among these two properties [30][31]. Cellular autoaggregation is directly proportional to the colonization of the gastrointestinal tract by LAB [32]. The results in the present study provide insights into the

effects of hydrophobic interactions on adhesion and adherence to Caco2 cell lines [33]. The pathogen adherence towards the intestinal walls was inhibited due to the cellular aggregate formation in probiotic and the adhesion to the intestinal epithelium. Furthermore, the coaggregation is caused due to the adhesion and aggregate formation among the pathogens which facilitates the clearance [34]. It is revealed from the data which is obtained from *in vitro* experiments that the aggregation percentage was considerably higher among isolates *L. gastricus* BTM7 and *L. paracasei* CD4 in comparison to all other strains, and the same was comparable statistically with *L. rhamnosus* GG reference strain ($p>0.05$). It is revealed from the microscopic observations of stained preparation of cells, that adherence of different strains towards Caco 2 cells is strain specific whereby the strains, BTM7 and CD4 strain adhesion was comparable with the standard strain adhesion. For every microscopic field the capability of adherence was noted as the number of cells in each field, and also the number of microorganisms adhered to each cells. Needless to say that the microscopic counts are open to errors because in microscopic field the LAB cultures, primarily Lactobacillus, exist in chains and are not evenly distributed. A diffused or localized pattern of adherence is specified by the LAB culture tests. Overall, the tests of adherence that was used in the present study indicated that the strain tested have high potential for colonizing the gut; however, the adherence may vary among different *Lactobacilli* species.

Mucin adhesion

The Probiotic LAB adhesion for the mucus layer of host is a significant attribute because the colonization of LAB gets affected through it within the host's intestine. The adherence efficiency of the isolates, which was assessed using pig intestinal mucin, was 35 to 70 % as shown in (Table no. 3.2). Among the tested strains, *L. plantarum* K84 around (35%) and standard strain *L. rhamnosus* GG, these two strains possessed the minimum and the maximum mucin adhesion percentages. Further the presence of mucus-binding (MUB) proteins among the isolates was confirmed by molecular analaysis targeting mub gene which confirmed the amplification 150 bp PCR product. The inconsistency of adhesion ability towards mucin can further be attributed to dissimilar range of interaction among the LAB and molecular structure of mucin carbohydrate

chains. Furthermore, *Lactobacillus* sp. has earlier been outlined to provide functionally lectin-like adhesion factors that may contribute towards the adhesion of acidic mucin carbohydrate chains. However, the involvement of factors, like extracellular matrix protein and pili, can never be ignored for the adhesion of LAB cultures to mucus [35].

Table 3.2: Characterization of adhesion-related properties of some probiotic isolates

Isolates	% Autoaggregation	% Hydrophobicity	Mucin adhesion (%)
***L. paracasei* CD4**	$35^{ef}\pm1.4$	$31^{cf}\pm2.8$	$53^{cd}\pm1.1$
***L. gastricus* BTM7**	$33^{ef}\pm0.8$	$32^{df}\pm2.3$	$75^{fg}\pm1.5$
***Brev. aydinogluensis* BTM9**	$26^{de}\pm1.8$	$23^{ac}\pm1.8$	$34^{a}\pm2.0$
***Brev. thermoruber* CD13**	$19^{bd}\pm1.2$	$25^{bd}\pm1.2$	$54^{cd}\pm1.5$
***Enterococcus sp* GTM14**	$31^{ef}\pm2.1$	$15^{a}\pm0.9$	$37^{a}\pm2.0$
***Brevi. thermoruber* HM29**	$31^{ef}\pm1.7$	$25^{bd}\pm1.4$	$42^{ab}\pm2.5$
***Brev. thermoruber* HM34**	$26^{ce}\pm1.3$	$28^{be}\pm1.1$	$40^{a}\pm1.5$
***W. confuse* CD1**	$29^{de}\pm2.5$	$21^{ab}\pm1.7$	$52^{d}\pm1.5$
***L. fermentum* K75**	$12^{ab}\pm1.9$	$37^{fg}\pm2.5$	$62^{de}\pm2.5$
***L. fermentum* K78**	$12^{ab}\pm1.6$	$34^{eg}\pm2.0$	$68^{ef}\pm1.1$
***L. plantarum* K84**	$14^{ab}\pm2.0$	$42^{g}\pm1.0$	$35^{a}\pm0.5$
***L. plantarum* K90**	$16^{bc}\pm1.6$	$30^{cf}\pm2.0$	$52^{d}\pm2.0$
***P. acidilactici* K94**	$12^{ab}\pm0.9$	$30^{cf}\pm1.5$	$41^{a}\pm1.5$
***P. acidilactici* K98**	$41^{f}\pm2.3$	$20^{ab}\pm1.0$	$70^{eg}\pm0.5$
***L. fermentum* K100**	$5^{a}\pm0.2$	$37^{fg}\pm2.0$	$51^{bc}\pm3.2$
***L. rhamnosus GG* LRGG**	$31^{e}\pm1.8$	$42^{g}\pm1.5$	$78^{g}\pm2.0$

"a-g Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)"

Adhesion to Caco 2 cell lines

The ability of *L. gastricus* BTM7, *L. paracasei* CD4, and a reference strain *L. rhamnosus* GG to adhere Caco2 cells was microscopically examined. The results of adherence studies indicated that stains *L. gastricus* BTM7 and *L. paracasei* CD4 exhibited the maximum adhesion to Caco2 cells whereby the cells are attached to cell lining of Caco2 cells (Fig. 3.1).

3.3.3 Functional properties

Antioxidant properties

The antioxidant activity of all the isolates, which was assessed using DPPH radical scavenging activity, was 35%–69% inhibition; whereas the natural standard, ascorbic acid exhibited 78% of scavenging activitiy. Oxidative stress, manifested as increased intracellular oxygen radical concentrations, can be attenuated by consuming antioxidant-rich diets. However, the anti-oxidant potential of LAB can be utilized in order to conquer the oxidative stress which is reported many research studies [36]. The activity of each isolate was in percentage range of 35 to 69 percent for DPPH and 45 to 92% ABTS free radical scavenging activities. *L. fermentum* K75 and *P. acidilactici* K94 expressed the maximum anti-oxidant activity of 92% that is resulted according to both of assays (Fig. 3.3). However, the mechanisms of probiotics and their ability of oxidation resistant are not very well understood. The suggested mechanisms underlying the antioxidant potential of LAB include production of anti-oxidant enzymes, metal ion chelation, antioxidant metabolite production, regulation of gut microbiota composition, anti-oxidase activity upregulation in the hosts, and down regulation of enzymes responsible of production of reactive oxygen species in the host. The free radicals property for scavenging through the cultures of food-grade has got the potential to be used in manufacturing of antioxidant rich products. They may act as additional health-enhancing antioxidants from dietary sources [37]. The antioxidant activities of the cell-free extracts of LAB have been previously reported [38][39]. However, the mechanisms behind these observations are not reported.

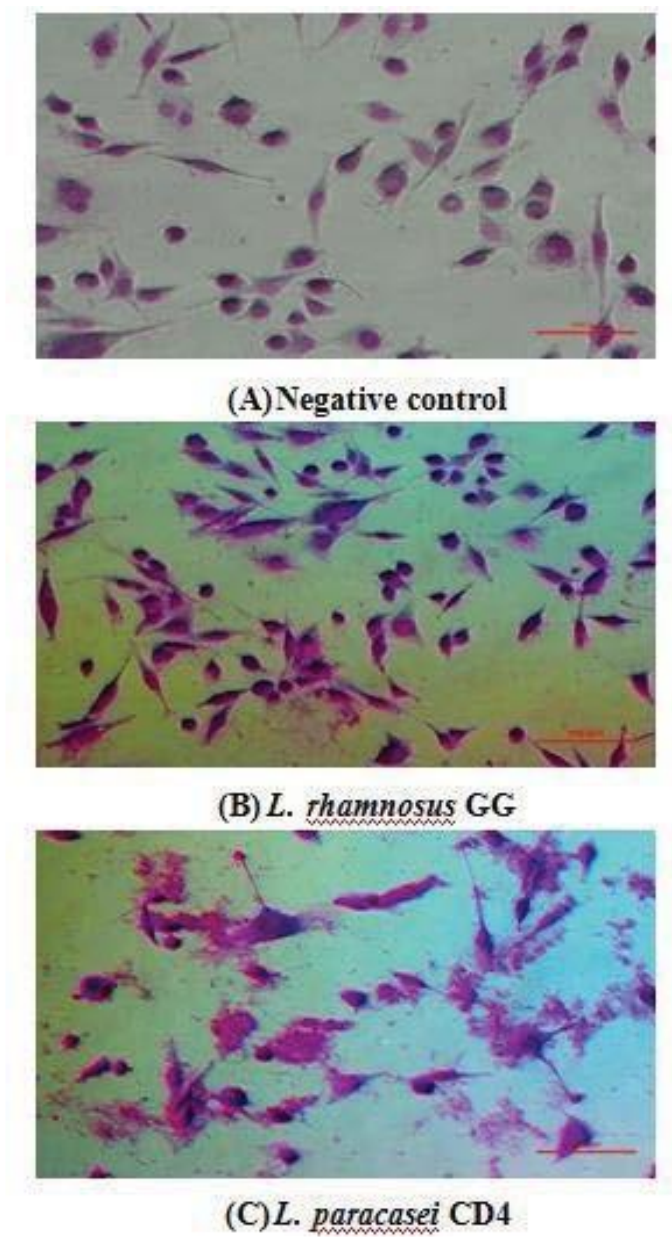


Fig 3.1: Adhesion of probiotic isolates to Caco2 cell lines

Table 3.3: Antioxidant potential of probotic isolates against free radicals

Antioxidant activity (% free radical scavenging)		
Isolates	**DPPH assay**	**ABTS assay**
***L. paracasei* CD4**	52.12^{d} ±2.00	68.02^{bc}±10.0
***L. gastricus* BTM7**	51.23^{cd}±1.00	75.23^{ab}±2.10
***Brevi. aydinogluensis* BTM9**	35.51^{a}±4.10	57.20^{ab}±1.50
***Brevi. thermoruber* CD13**	40.22^{ab}±2.30	47.14^{a}±0.55
***Enterococcus sp.* GTM14**	43.12^{ac}±2.10	49.15^{ab}±2.10
***Brevi. thermoruber* HM29**	45.51^{bd}±2.60	50.13^{ab}±0.60
***Brevi. thermoruber* HM34**	40.32^{ab}±2.20	45.20^{a}±1.10
***W. confuse* CD1**	36.25^{a}±3.30	45.10^{a}±0.40
***L. fermentum* K75**	68.17^{e}± 1.19	92.88^{d}± 0.32
***L. fermentum* K78**	65.49^{e}± 0.54	88.88^{cd}± 1.49
***L. plantarum* K84**	64.98^{e} ± 1.00	90.67^{d}± 0.43
***L. plantarum* K90**	63.38^{e} ± 0.55	90.77^{d}± 0.40
***P. acidilactici* K94**	64.52^{e}± 2.04	92.89^{d}± 0.64
***P. acidilactici* K98**	69.11^{e} ± 0.93	90.37^{d}± 0.27
***L. fermentum* K100**	63.13^{e} ± 0.96	87.94^{cd}± 1.36
***L. rhamunosus* GG**	67.26^{e} ± 0.76	87.62^{cd}± 0.58

"a-h Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)"

BSH and cholesterol-lowering activity

All the probiotic isolates tested resulted in positive response for BSH activity, which was ascertained around the colonies as a white precipitate on bile-supplemented agar plates (Fig. 3.2). An amplicon of 250 bp was obtained targeting the bsh genes which further confirmed the BSH activity. The bile salts deconjugation is caused due to the detoxification of bile salts by hydrolase activity; the concentration of liberated free primary bile acid was determined qualitatively as white precipitation surround the colonies [40]. Furthermore, with the help of bsh primers an amplicon of 250 bp was obtained, which thereby confirmed the activity of BSH among isolates. The isolates relatively lenient to bile salts may render because of the presence of BSH activity, which further leads to reduced level of blood cholesterol in the host [41] mentioned in (Table 3.4). As listed with the presence of BSH activity, cultures could be helpful in controlling the serum levels of cholesterol, which is either due to incorporation of cholesterol into or their cell walls adhesion or reduction of enzymatic activities of cholesterol reductase. In the present study, the results indicated that *L. plantarum* K90 possessed the highest potential in cholesterol-lowering activity ($p<0.05$). These effects of lowering of cholesterol are primarily attributable to the activity of BSH among the probioticcultures. However, contributions of other mechanisms, like assimilation of cholesterol through bacterial cells, the binding of cholesterol to bacterial cell walls, or physiological actions of short-chain fatty acid fermentation for the end products, must be considered.

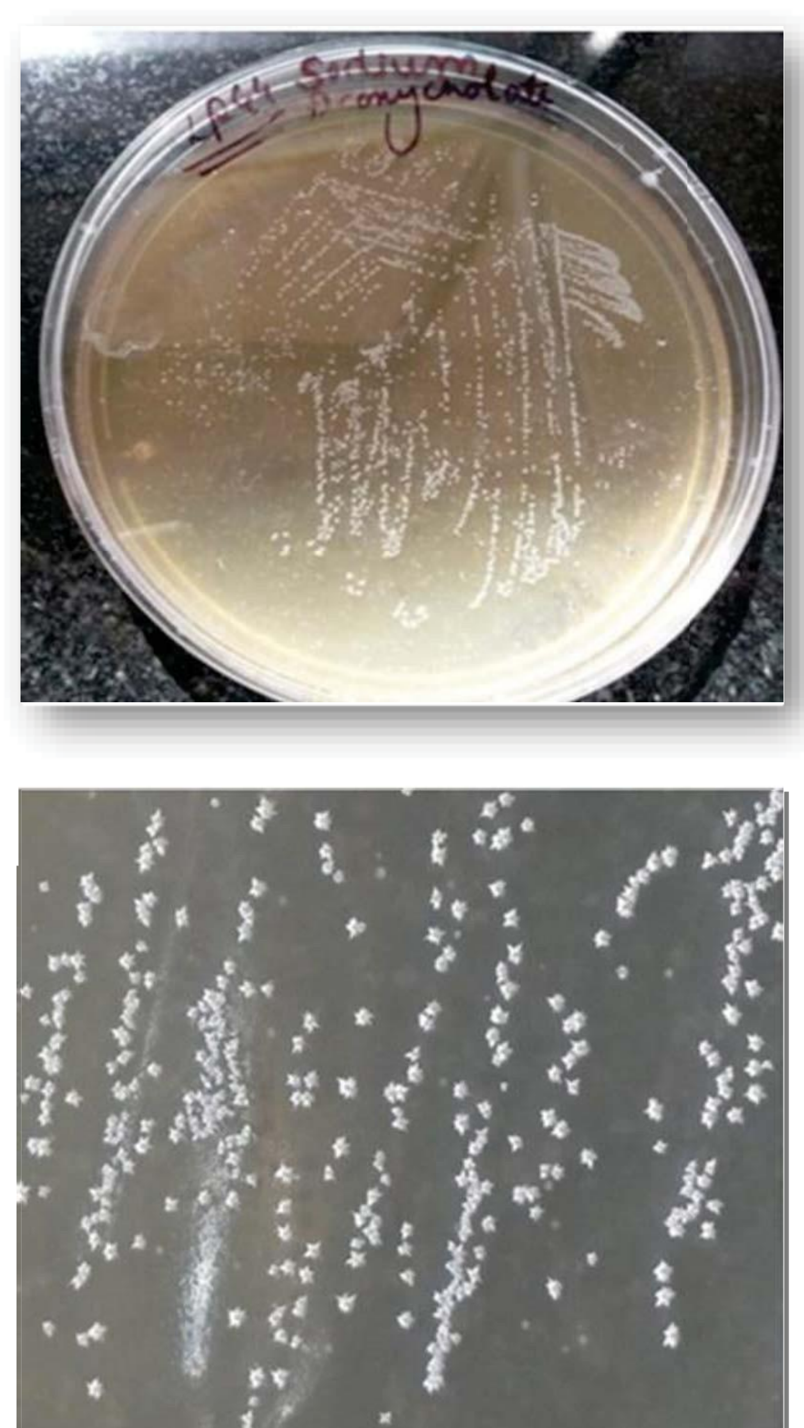


Figure 3.2: Precipitates indicating the bile salt hydrolase activity in probiotic isolates

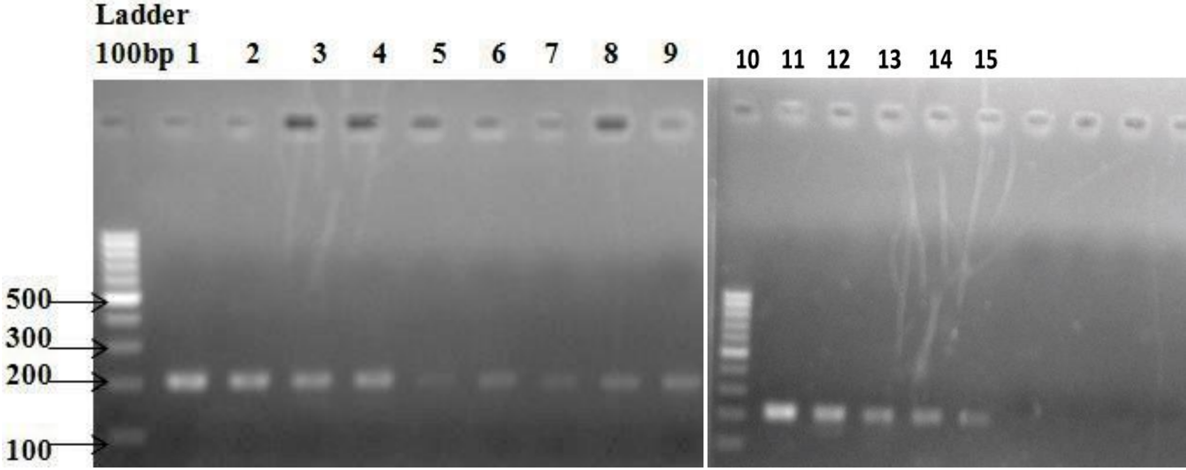


Figure 3.3: Bile salt hydrolase activity with an expected amplicon length: 205.

"1, *L. paracasei* CD4; 2, *L. gastricus* BTM7; 3, *Brev. aydinogluensis* BTM9; 4, *Brev. thermoruber* CD13; 5, *Enterococcus sp* GTM14; 6, *Brevi. thermoruber* HM29; 7, *Brev. thermoruber* HM34; 8, *W. confuse* CD1; 9, *L. fermentum* K75; 10, *L. fermentum* K78; 11, *L. plantarum* K84: 12, *L. plantarum* K90; 13, *P. acidilactici* K94; 14, *P. acidilactici* K98; 15, *L. fermentum* K100"

Exopolysaccharide (EPS) production

After the three days of incubation of all the probiotic cultures on MRS agar supplemented with 1% sucrose, mucoid colonies were obtained. Therefore, the production of EPS through LAB was confirmed (Table 3.4). The EPS production is very well recognized in LAB used in fermentation as the production of EPS by these cultures provides a high viscosity as well as desirable texture and consistency to the product. As we have used the fermented dough for isolation of lactic cultures, the isolates were presumed to have synthesized a wide variety of structural exopolysaccharides, such as glucans or fructans [42]. The EPS production might have attributes of prebiotic which is considered with fructan's bifidogenic effect produced *by L. sanfranciscensis.*

Table 3.4 Functional attributes of probiotic isolates including, bile salt hydrolase activity, cholesterol lowering property and exopolysaccharide production

Isolates	**BSH activity**	**Cholesterol lowering activity (%)**	**EPS**
L. paracasei CD4	+	69.12^{gh}±2.0	+
L. gastricus BTM7	+	74.23^{h}±2.0	+
Brevi. aydinogluensis BTM9	+	43.21^{ab}±2.5	+
Brevi. thermoruber CD13	+	42.20^{ab}±2.5	+
Enterococcus sp. GTM14	+	50.13^{bd}±1.0	+
Brevi. thermoruber HM29	+	39.91^{a}±1.5	+
Brevi. thermoruber HM34	+	42.63^{ab}±2.5	+
W. confuse CD1	+	59.08^{ef}±1.1	+
L. fermentum K75	+	65.93^{fg}±1.5	+
L. fermentum K78	+	55.82^{ce}±0.5	+
L. plantarum K84	+	59 $.23^{ef}$±1.1	+
L. plantarum K90	+	75.62^{h}±1.5	+
P. acidilactici K94	+	58.31^{df}±2.0	+
P. acidilactici K98	+	49.46^{bc}±2.5	+
L. fermentum K100	+	50.29^{bd}±0.5	+
L. rhamunosus GG	+	61.24^{eg}±1.5	+

+ presence of BSH activity and EPS production

“a-h Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey’s – post-hoc range test between replications. ($p<0.05$)”

3.4 Conclusion

A total of 15 isolates belonging to group of lactic acid bacteria were obtained from dairy and cereal based foods. These isolates were able to fulfill the criteria of probiotic as per DBT and ICMR guidelines. The strain specific activities were obtained for adhesion potential of the strains. Apart from the probiotic attributes, the cultures also possessed functional activities such as antioxidative and cholesterol lowering activities. These *in vitro* studies confirmed the probiotic potential of these isolates which needs to be further confirmed in *in-vivo* models.

Chapter 4

To study the effect of selected isolates for probiotic properties using model system *Caenorhabditis elegans*

4.1 Introduction

Probiotic lactic acid bacteria (LAB) are accounted for managing intestinal health of host by controlling intestinal microflora specifically or not specifically and usually perceived as secure, Generally Recognized as Safe (GRAS) for the fermentation of food and feed. Most of the probiotic studies till date have been examined from regular food items of dairy, vegetables, natural items, meat and, gastrointestinal tract of human [1]. In order to present the health advantages, the capacity of probiotics for the colonization of the gastrointestinal (GI) tract and the capability of probiotics and to stay in the GI tract is essential criteria for a probiotic candidate [2], [3], [4] [5]. For selection of probiotic strains, the adherence potential and colonization has been studied in in vitro models, such as the adherence to the intestinal cells of human (HT-29 and Caco-2 cells), and adhesion tests for mucin [6], [7] and [8]. In order to expand the probiotic candidate selection efficiency, *C. elegans* termed as free-living bacterivorous nematodes in natural and agricultural soil, has been proposed as in model of *vivo* screening of probiotics. The demonstrated model is one step nearer towards framework of multi-cellular animal with integral intestinal microbiota [9]. Furthermore, the investigations in this nematodes model present a basic, fast, and cost-effective selection of potential probiotic microorganisms [10] which would have laborious and time-consuming selections through mice model studies. The additional favorable preferences for utilizing *C. elegans* include: transparent body, which permits clear perception of each cell at its distinctive progressive stages [11], and the structure of intestinal cells which is comparatively same as in humans [12]. Because of these characteristics, the *C. elegans* has been effectively utilized as a suitable model to learn anti-infective, anti-oxidative and life expectance expanding (longevity) effect of lactobacilli [13].

From our past investigations, we have obtained a collection of fifteen different probiotic candidates isolated from fermented dairy or cereal based foods. All those cultures resulted in strain specific probiotic qualities as dictated *in vitro* test assays in chapter 3 of this thesis [14]. In order to present the adhering capability of these cultures and for colonizing the layers of intestine, the current investigation was intended to evaluate their impact on life expectancy and the colonization in *C. elegans*. The prime goal of this current examination was to utilize the *C. elegans* life-span test model for measuring the responses of the worms for indigenous probiotic cultures and to monitor the colonization potential of these cultures in intestinal wall of the worm.

4.2 Materials and methods

4.2.1 Cultures and their maintenance

The pure cultures of the fifteen isolates were activated from their glycerol stock in MRS broth

C. elegans (Bristol N2) strain of wild type (from CGC, University of Minnesota, USA) was used

The worms were maintained on NGM (Nematode growth medium) plates which were seeded through *E. coli* OP50, uracil auxotroph which is a conventional food source for *C. elegans* at the temperature of 20 °C

The gravid nematodes that were bleached through the mixture (bleach:potassium hydroxide 5 M (1:1)) were utilized in order to obtain the age synchronized L4 nematode for assay

The age synchronized worms were grown on newly seeded NGM plates for 2 days before the start of the experiment

4.2.2 Liquid killing experiment of worms fed with probiotic isolates

- The young adult worms (~10) were moved from NGM plates that were removed through the lawn of *E. coli* OP50.
- The worms were cleaned by washing them three times utilizing the pure M9 buffer solution that contains (3 g/L KHPO4, 5 g/L NaCl, 6 g/L Na2HPO4, 1 ml 1 M MgSO4) [13].
- The washed worms were transferred in 24 well plate, containing M9 buffer with overnight grown probiotic or *E. coli* OP50,
- Before adding the probiotics to the wells, the OD_{600nm} of the broth was set to 0.5.

- The inoculated plates were incubated for 7-10 days at the temperature of 20°C
- At regular intervals of each day, variation among morphological, physiological, survival and life expectancy of C. *elegans* fed through LAB, was monitored.
- The parent worms were exchanged to another well in periodic times after each two days in order to isolate them through the progenies.
- The worms were found as dead whenever there is no any sign of improvement or any response towards outside stimulus (fragile touch through a platinum loop).
- The test was done in triplicates and percent survival of the worms was recorded. [9].

4.2.3 Microscopic observations of worms

- The changes in morphology and physiology of worms fed with probiotic isolates were observed on daily bases.
- The grown-up young adult worms were withdrawn from plates and washed twice by M9 buffer. The cleaned worms were transferred to fresh clean tube.
- The worms were anesthetized using M9 buffer containing sodium azide (1mM) for few minutes.
- The worms were placed on clean glass slide and observed under NIKON SMZ1000 stereomicroscope.
- Finally, the worms afterwards analyzed for their integral inner shapes, generation and eggs creation as the sign of healthful worms [16].

4.2.4 Binary Choice assay

- A binary choice test was performed to observe the worm's preference towards different probiotic microorganisms as an alternate food to *E. coli* OP50.
- The test was conducted on NGM plates using standard protocols
- The probiotic cultures and ***E. coli*** OP50 were grown overnight in MRS broth and LB broth, respectively to an OD of 0.5

- Both the cultures were spotted on plates of NGM at zone A with probiotic anD Zone B with *E. coli* OP50 at B (zone) through the separation of around 3 cm from the middle.
- A total of 25 worms of L4 stage, cleaned by washing were placed in the middle of the plates with equivalent separation among zone A and B.
- After the four hours of incubation at the temperature of 20º C, the worms were counted in both zones. For zone A and B, the plate which contains OP50 were considered as control [18].
- The analysis was performed three times and the calculation for binary choice was calculated as:

$$\text{Choice of index} = \frac{\text{Worms quantity in tested bacteria} - \text{Worms quantity in } E.coli \text{ OP50}}{\text{Total amountof worms}}$$

When CI corresponds to negative 1, it shows full probiotic disinclination and inclination for *E. coli* OP50, on the other hand when CI corresponds to positive 1, it shows full inclination towards test microorganism, and when CI corresponds to 0, it shows an equivalent distribution.

4.2.5 Pharyngeal pumping assay

In order to evaluate the physiological responses of the worms towards probiotic addition, the movement for pharynx the pharyngeal pumping test was done.

- For this purpose, the probiotic cultures were seeded over the worms of L4-stage in NGM plates as described in above section
- The plates were incubated at temperature of 20°C. After every 24 h interval, pharyngeal pumping rate was counted upto 7 days of incubation.
- The number of contractions was counted in order to determine the pumping rate among pharynx terminal bulb for 10 successive seconds.

4.2.6 Intestinal colonization of LAB in *C. elegans*

- The worms around (n=25) were incubated in the wells containing probiotic cultures ($OD_{600\ nm}$ =0.5) for measuring the intestinal colonization of different cultures in *C. elegans.*

- The plates containing probiotics and worms were incubated as described in above section for 24-72 h.
- After a regular interval of 24 h, the worms were removed from the wells and washed with M9 buffer for microscopic observations.
- In a microcentrifuge tube of 1.5 ml, the worms were crushed in M9 buffer and serial dilutions in normal saline were prepared.
- The serially diluted suspensions were plated on MRS agar for determination of viable cell counts of probiotic cultures
- Fluorescent microscopy was used for analyzing the *in vivo* colonization of probiotic isolates within *C. elegans*
- The washed worms were stained with acridine orange of (0.1 %) for 15 min.
- The stained slides were observed for adhesion to intestine of *C. elegans* using Eclipse Ti-S of NIKON, Japan.
- The amount of fluorescent produced was taken as directly proportional to the intestinal LAB colonization [18].

4.2.7 Statistical analysis

All the tests were done in triplicate and the data was represented as average of three replications. ANOVA and Turkey's tests were used for comparison analysis at $p < 0.05$ to determine the effect of different probiotic strains on host using SPSS [20].

4.3 Results and discussion

4.3.1 Effect of probiotics on life span of *C. elegans*

For examining the life span and aging related processes, the C. *elegans* has been accounted as an appropriate model organism because C. *elegans* follows progressive conserved metabolism and host defense system as for the human hosts [21]. To determine the efficacy of our probiotic

isolates on *C.elegans* model system, a liquid killing assay was conducted to test survival, physiological and morphological alterations in *C. elegans* when the worms were treated with probiotic bacteria. The results of these survival assays are presented in Fig 4.1. The results obtained on these assays indicated that the life expectancy for *C. elegans* was increased with addition of probiotic cultures as food source. Among the tested strains, *L. paracasei* CD4 demonstrated the highest survival percentage of worms when compared with the standard food *E. coli* OP50 taken as control. The lowest rate of survival was observed for *P. acidilactici* K98 ($p<0.05$). During the regular monitoring of the survival for 7-10 days, 40 % of worm's survival was observed in standard strain *L. rhamnosus* GG which resulted in complete killing of the worms after 10 days of incubation. On the other hand, *L. plantarum* K90, *L. gastricus* BTM7, *L. paracasei* CD4, enhanced the life expectancy by five days (Fig. 4.2). Each of worms possessed normal growth, improved intestine system and laying of eggs through ordinary reproduction by considering all other morphological and physiological changes Fig 4.3.

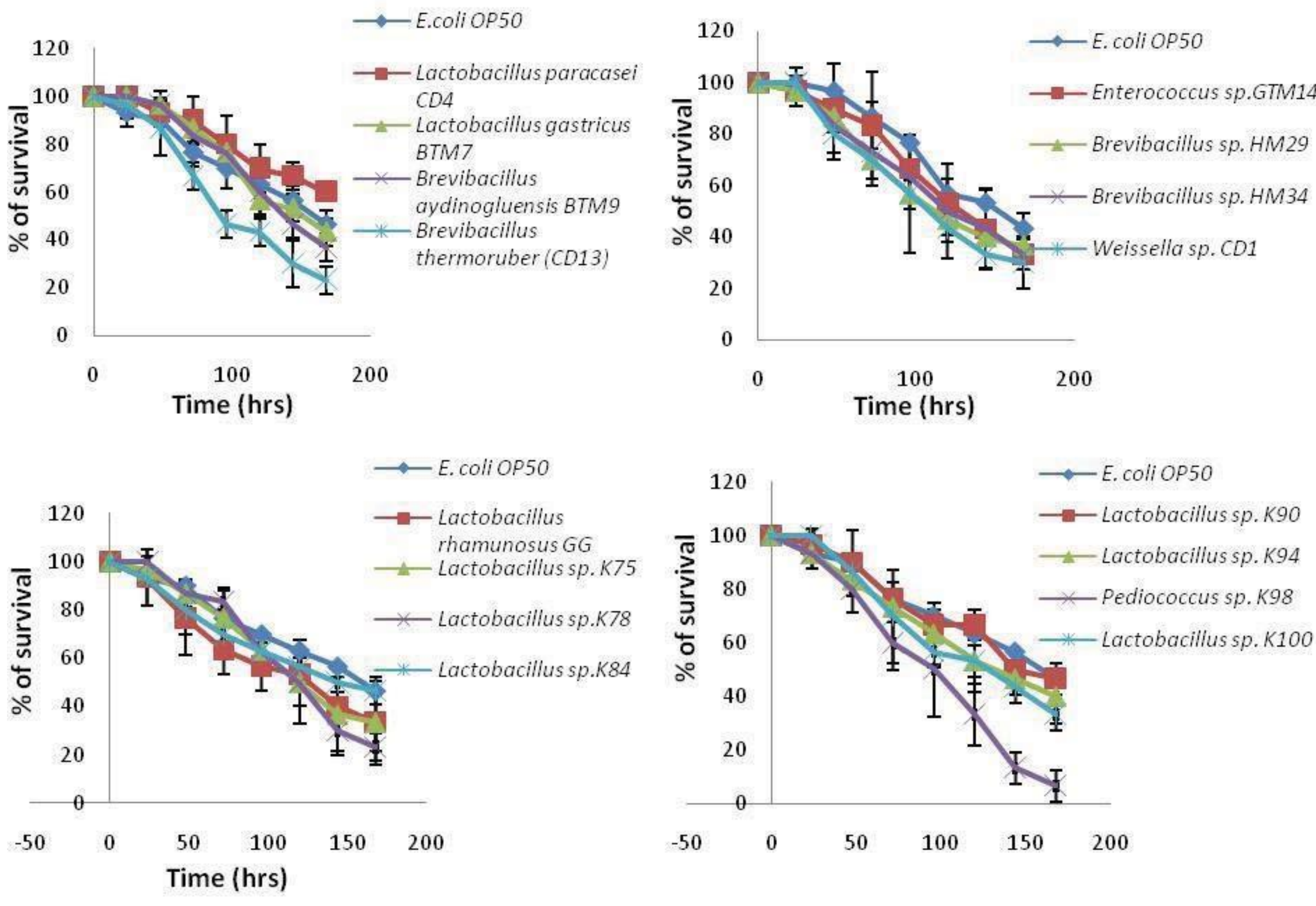


Figure 4.1 Percent survival of worms treated with probiotic isolates in liquid culture

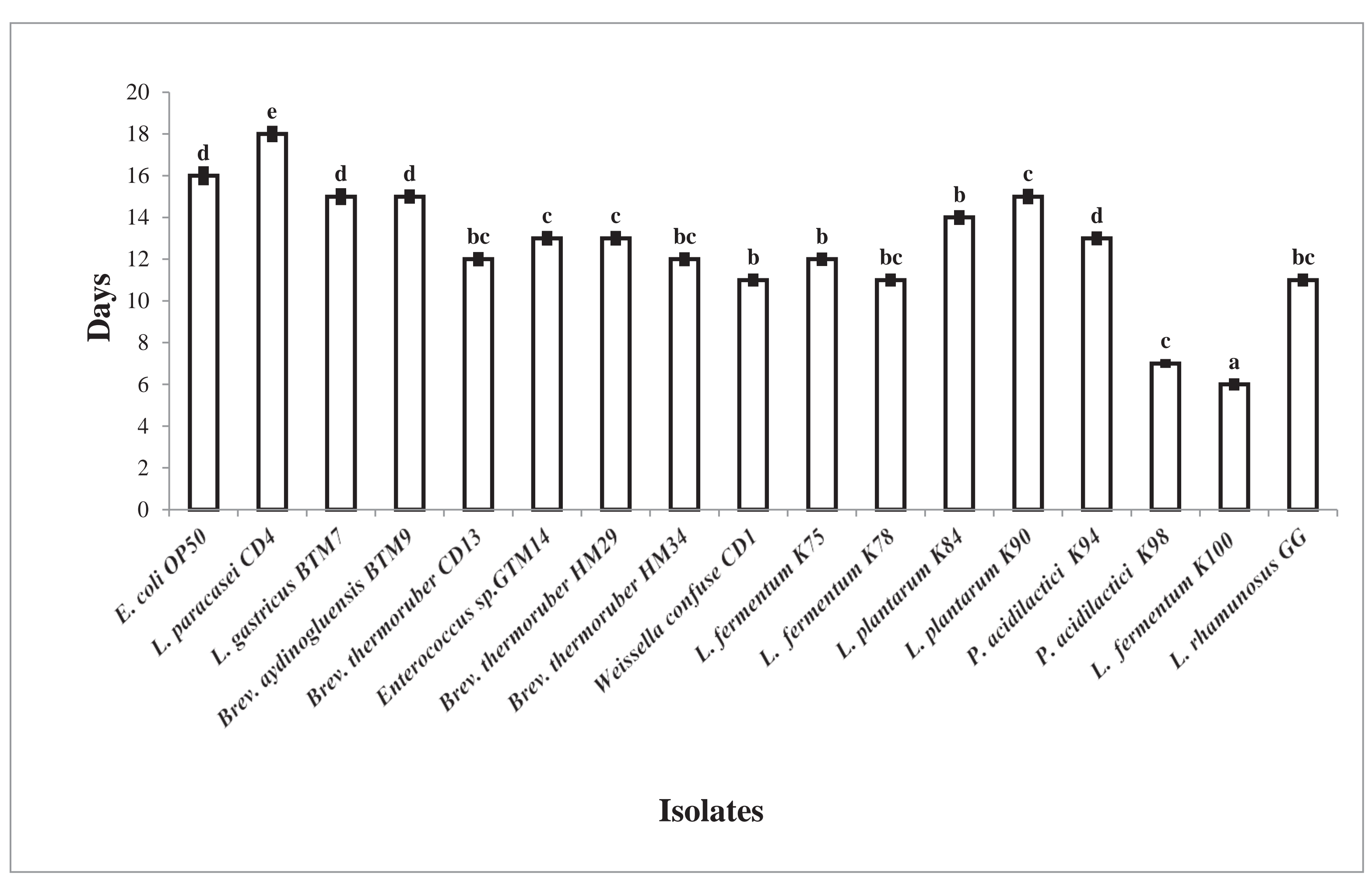


Figure 4.2: Mean Life Span

“a-e Means in the bars with same superscript letter are not significantly different as measured by 2 sided Tukey’s – post-hoc range test between replications. (p<0.05) “

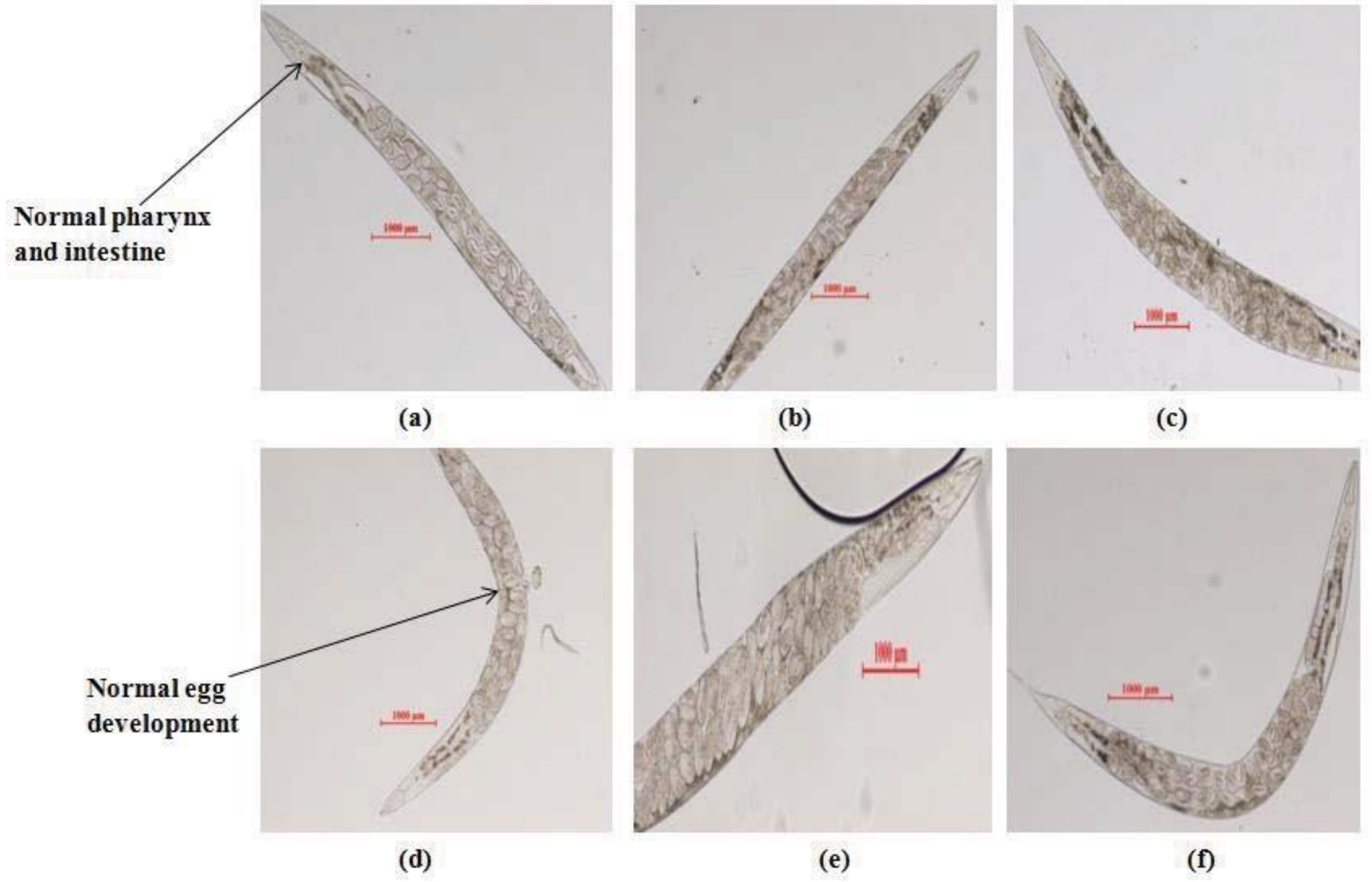


Figure 4.3: Microscopic images depicting physiology and morphology of *C. elegans* administered with different probiotic isolates lactic acid bacteria. "a) *L. paracasei* CD4 b) *Brev. aydinogluensis* BTM9 c) *Enterococcus sp.* GTM14 d) *L. plantarum* K84 e) *L. rhamnosus* GG f) *E. coli* OP50 (control)"

In early 1907, Metchnikoff reported that the lactic acid bacteria play an important role in maintaining the health of humans and discussed the long-life span of human those who used to eat a lot of yogurt [22]. The similar mechanisms might play a vital role in the results obtained in the current investigation showing that the comparative enhancement of life span through lactic cultures added as food source for the *C. elegans*. Even though, the limitations in diet was recognized to expand the life expectancy among various species, along with worms, rodents, yeast and probably primates [23], however, this was not observed in our study. In various other studies on the ageing phenomena in the worms, restricting calories and resistance from stress were described as possible mechanisms behind increased life expentencies. Furthermore, for the resistant from stress, the JNK-1/DAF-16, P38 MAPK/SKN-1 and DAF-2/DAF-16 pathways were reported in increasing the life span of the worm [24]. Few studies have also reported the increased life span of *C.elegans* on addition of the probiotic lactic acid bacteria [9], [25], [26], [27], [28]. These studies concluded that the immunomodulatory activities of LAB and their metabolites are helpful in upgrading the life span of *C. elegans* and having distinctive advanced effects of health on host. The improvement in movement of body, body size reduction and lessoning of lipofuscin accumulation were also marked as the most vital biomarkers for ageing in [29], [30], although these were not studied in the present investigation

4.3.2 Binary choice assay

As from the results of survival assays, it was observed that all the lactic cultures tested in the present investigation did not possess any lethal effect on the worm, therefore, these probiotics were further tested as a possible food preference in comparison to the standard *E. coli* OP50. The preference of food choice is determined by calculating the binary choice index. The results obtained in the binary choice tests indicated that the worms displayed inclinations towards particular cultures of probiotics Fig. 4.4. The outcomes also suggested that the increasing effect of life span of lactic cultures was not because of any restrictions in dietary as the worms prefer to use probiotics as their food. The results demonstrated that through initial hours of incubation, the quantity of worms in both the zones was found to be roughly equivalent. However, in the later period of incubation, about 80 to 90 percent of worms were observed utilizing *L. gastricus* BTM7, *L. plantarum* K90, *Brev. thermoruber* HM29 and *L. paracasei* CD4 as preferred food source in comparison to *E. coli* OP50t. Additionally, for rest of tested strains the choice of index was also observed as negative one (-1). Recent literature studies have reported the role of

diacetyl, which is developed as a product of fermentation for LAB, in order to know its chemoattraction capability with the help of odor receptors [31]. The sensitive olfactory neurons possessed by nematodes detects different odors demonstrates aversion, attraction and odor's memory [32].

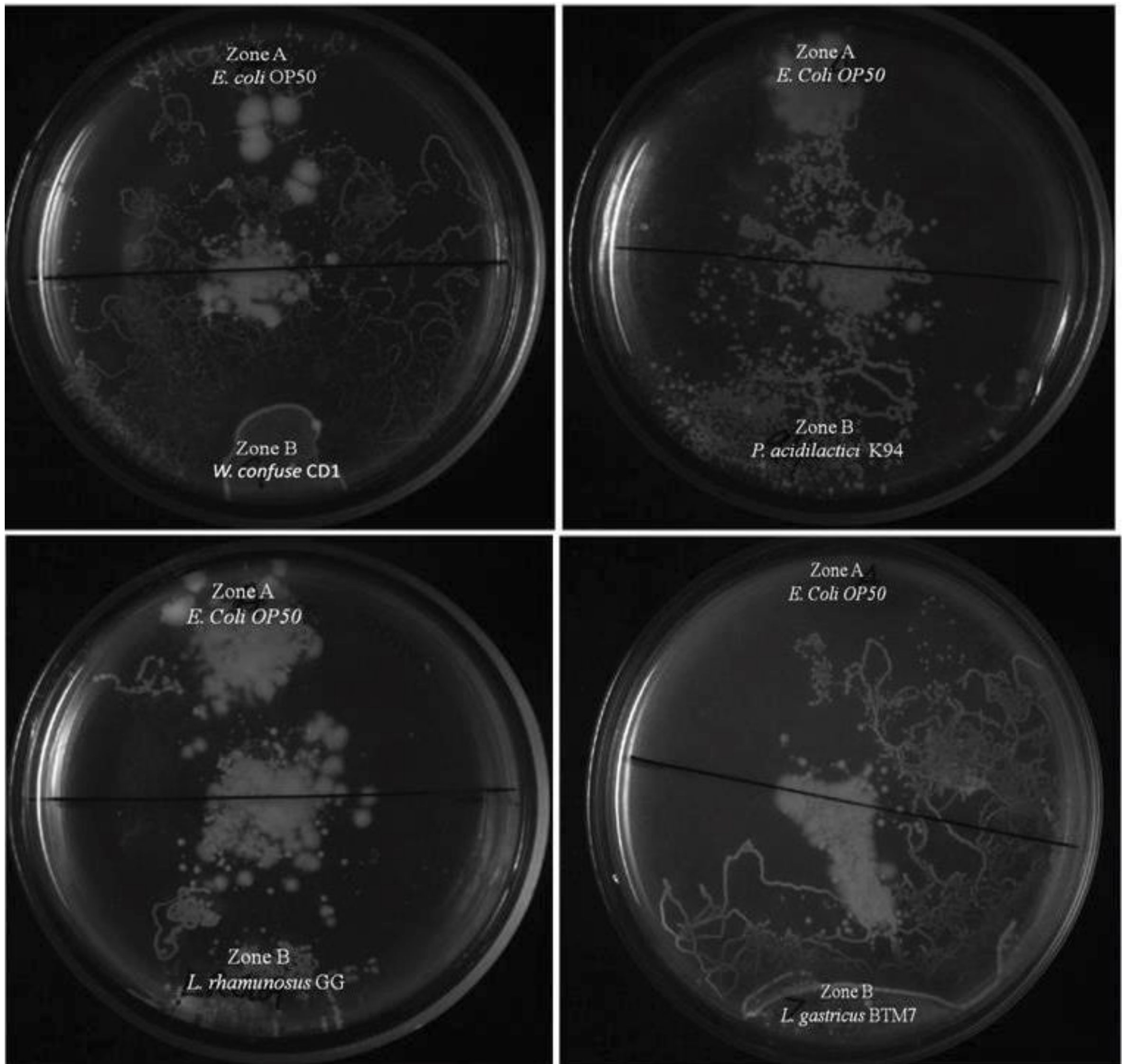


Figure 4.4: Binary Choice assay for food preference of *C.elegans* towards probiotic cultures, *E. coli* OP50 marked as zone A and LAB as zone B. The worms were allowed to move freely on the agar plate for 24 h to track the path of *C. elegans*.

4.3.3 Pharyngeal pumping assay

The efficient eating and transfer of the food in intestine, becomes helpful through the pharyngeal pumping. The pharyngeal component of *C. elegans* comprises of three various practical units that are isthmus, corpus and terminal bulb. The feeding behavior of worms was characterized by the relaxation and contraction of pharynx, where the pharynx behaves as the neuromuscular pump. The pump associated by electrically coupled muscles which pumps throughout life of the *C. elegans* [33]. The results obtained for the effect of probiotics on pharyngeal pumping indicated that the tested probiotics did not have any significant effect on the rate of pharyngeal pumping. The pharynx was intact and after the incubation for the duration of seven days it was observed that the pharyngeal pumping rate was nearly equivalent to standard food of *E. coli* OP50 taken as control. Despite of the fact, that pharyngeal pumping frequency for most of the worms declined with age, and after the incubation for 10 days there was no any major change found in worms that were fed with the probiotic cultures in comparison to the worms that were fed with *E. coli* OP50. The lactic cultures may activate the serotonin action which is suggested as an endogenous pharyngeal pumping and isthmus peristalsis activator by enacting neural pathways and G12 and SER-7 alpha signaling pathways [34]. Another study reporting the normal pharyngeal pumping on addition of probiotics, indicated the role of restriction in dietary for initiating the long life span of C. *elegans* by the decrease in pumping rate with *L. salivarius,* though it was not observed in our study. The reduction in caloric intake may be induced by the decrease in pumping rate that further effects the growth of worm.

4.3.4 Intestinal colonization assay

The health promoting abilities of the probiotics are reported as they are able to colonize the host intestinal lining. As a result of this property, the colonization and the adherence were the foremost requirement for selection of potential strains [36]. However, the capability of lactic cultures in adhering the gut of human (lumen) has been investigated for *in vitro models* by utilizing the cell cultures of animals. There exist very scanty reports on use of C. *elegans* for colonization and adherence of probiotics in intestine. Additionally, the reports on natural gut microbiota of the C. *elegans* has also not been investigated in detail so far. Many studies have reported the symbiosis and commensalism between the microflora of intestine occur in the worm. Therefore, we have determined the efficacy of our strains, to adhere and colonize the

intestine of the worm by incubating the probiotics with worm for different time intervals. At each time points, the worms were harvested and investigated for microbial viable cell count and fluorescent microscopic observations of intestine lining Table 4.1. The results obtained in the study indicated that with the increase in the duration of incubation for around 72 h, the bacterial colonization was higher. The worms started colonizing the intestine after 24 h which tends to increase with the time fo incubation. An average count of $\log_{10}$ 4 to 6 CFU per worm was observed for all the probiotics tested, however the colonization potential was strain specific as different cultures resulted in diverse capability of colonization. The microscopic observations also indicated the increased fluorescence with the increase in time of exposure to probiotic cultures Fig. 4.5.

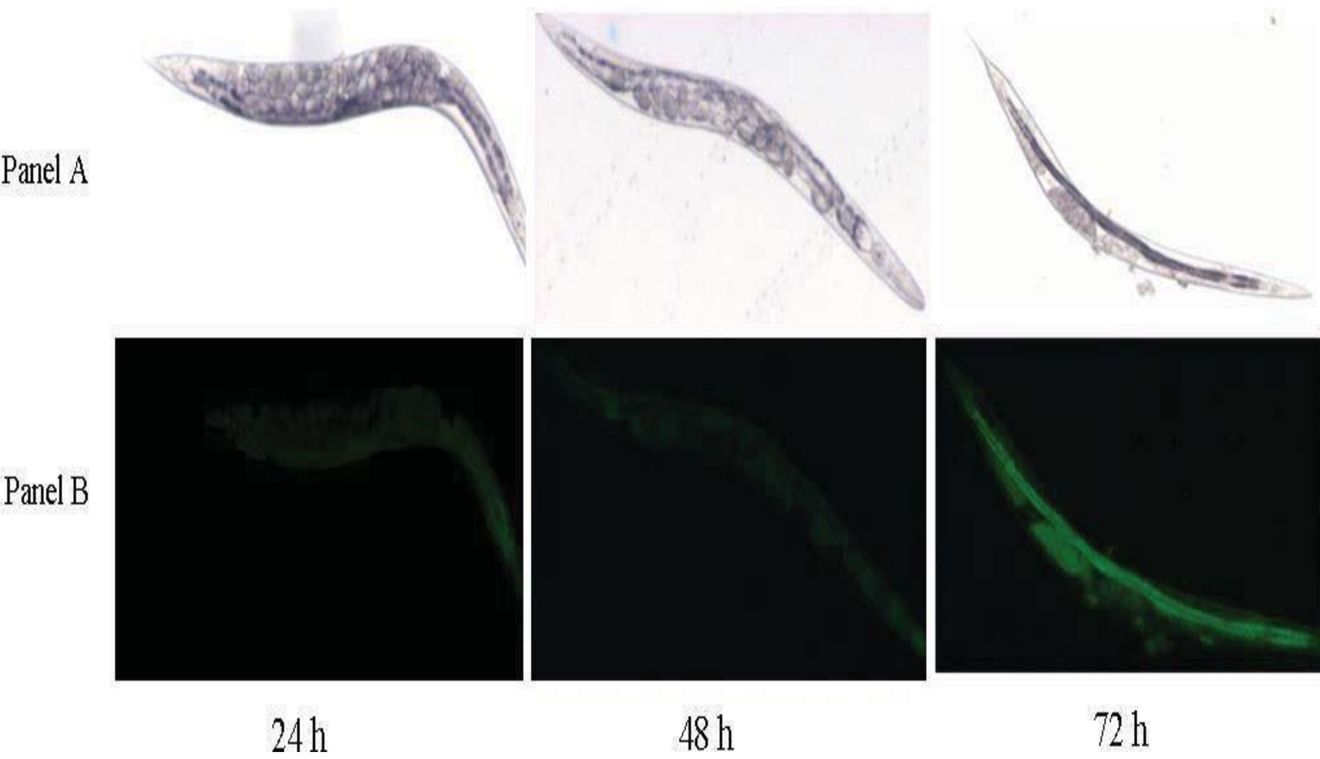


Figure 4.5: Microscopic images of *C. elegans* administered with *Lactobacillus gastricus* BTM7 at different time intervals. Stereo microscopic (Panel A) and fluorescent microscopic (Panel B)

Table 4.1: Viable Cell counts of probiotic cultures (Log_{10} CFU per worm) in the *C. elegans* intestine during 72 h of incubation

Isolates	24 h	48h	72h
L. paracasei CD4	$5.25^{cd,x}$	$5.30^{b,x}$	$5.5^{de,x}$
L. gastricus BTM7	$4.00^{ab,x}$	$4.30^{a,x}$	$5.00^{ab,y}$
Brevi. aydinogluensis BTM9	$5.27^{cd,x}$	$5.36^{b,x}$	$5.53^{de,x}$
Brevi. thermoruber CD13	$5.60^{d,x}$	$5.20^{b,x}$	$5.40^{de,x}$
Enterococcus sp. GTM14	$5.23^{cd,x}$	$5.30^{b,x}$	$5.41^{de,x}$
Brevi. thermoruber HM29	$4.60^{b,x}$	$5.04^{b,y}$	$5.20^{bcd,z}$
Brevi. thermoruber HM34	$4.84^{c,x}$	$5.00^{b,x}$	$5.11^{bc,x}$
W. confuse CD1	$4.47^{ab,x}$	$5.07^{bc,y}$	$5.41^{de,y}$
L. fermentum K75	$5.07^{cd,x}$	$5.40^{b,y}$	$5.55^{de,y}$
L. fermentum K78	$5.00^{cd,x}$	$4.47^{b,y}$	$5.00^{ab,y}$
L. plantarum K84	$5.11^{cd,x}$	$5.17^{b,x}$	$5.25^{bc,x}$
L. plantarum K90	$5.20^{cd,x}$	$5.40^{b,x}$	$5.60^{e,x}$
P. acidilactici K94	$5.30^{cd,y}$	$4.69^{a,x}$	$5.07^{ab,xy}$
P. acidilactici K98	$5.0^{cd,x}$	$5.17^{b,x}$	$5.46^{cde,x}$
L. fermentum K100	$4.00^{ab,x}$	$4.30^{a,x}$	$5.00^{ab,y}$
L. rhamunosus GG	$4.30^{ab,x}$	$5.00^{b,y}$	$5.07^{ab,y}$

"a-f Means in the column with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)"

"x-z = Means in the row with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications."

4.4 Conclusion

The *C. elegans* can be used as an efficient in vivo model to screen the probiotics for their effect on life span and intestinal colonization. All the probiotics tested were able to colonize the intestine of the worm and increased the life span of the worms by five to six days without any alterations in the physiology of the worms. The probiotics also served as better food choice for the worms as indicated by higher binary choice index. From the tested strains, two of isolates that are *Lactobacillus plantarum* K90 and *Lactobacillus gastricus* BTM7 were selected for further investigations.

Chapter 5

To study the protective effect of probiotics against infection with *Cronobacter sakazakii* in *Caenorhabditis elegans*

5.1. Introduction

C. sakazakii is known to be gram negative, rod shaped opportunistic pathogen and an occasional contaminant in powdered infant formula. As food for neonates is prepared from powdered infant formula, these infant products may be a delivery vehicle of pathogen and its infections. The pathogen usually cause meningitis, bacteremia and necrotizing enterocolitis in neonates. Although the cases of infection of *C. sakazakii* are rare, however, the infection is reported to have a fatality rate ranging between 40- 80% as reviewed by [1]. The intermediate sensitivity has been described for the *C. sakazakii* against different stress such as osmotic and desiccation stresses. The tolerance of the pathogen to these stresses is reported due to its ability to form the biofilms. The cells in its biofilm niche are physiologically very distinct from their planktonic counterparts whereby these cells function as a cooperative consortium [2]. Due to its ability to form biofilm, it is very difficult to eradicate the colonization of the pathogen on biotic as well as abiotic surfaces. A wide array of chemical disinfectants and heat treatments have been employed to eradicate the biofilm formation by the pathogen however, strains are able to overcome this stress. To overcome this, biocontrol measure is one possible alternate suggested which could ensure that the foods and their surfaces are free from contamination. Few reports similar to [3] have reported the antimicrobial activities of bioactive peptides as fermentation product by *Lactobacillus acidophilus* against pathogenic bacteria-like *Cronobacter* sp.

The antimicrobial activities of probiotics against a wider strain of pathogens has attracted the interests of researchers with a possible application of potential probiotics as a bio preservative to inhibit the growth of food borne pathogens. These probiotics are generally recognized as safe (GRAS) therefore these are easy to incorporate in the food systems [4]. Lactic Acid Bacteria (LAB) is one of the most commonly used probiotic group of microorganisms and have been found to have a variety of physiological beneficial effects in the human hosts. The probiotics are reported to possess antimicrobial activity, antitumor effects, reduction of serum cholesterol, supplementary effects on nutrition and lipids, and immunomodulatory effects [5]. The antimicrobial activities of probiotics are mainly attributed to their ability to produce various inhibitory metabolites as described in chapter 2 of the thesis. Apart from metabolic products, the LAB also produces bacteriocins, which are proteinaceous compounds that possess inhibitory activities towards both Gram-positive as well as Gram-negative foodborne pathogens [6]. The

bacteriocin producing LAB strains are found in a wide range of host environments including fecal samples of humans and animals, various food products [7].

To identify the effective probiotics from different sources, selection through *in vitro* and *in vivo* studies usually is required [8]. Therefore, to identify the probiotic properties of LAB cultures *in vivo*, we have used *Caenorhabditis elegans* as an *in vivo* screening nematode model as reported in the previous objectives. The worm is well-studied biological system, commonly used as a model to study aging and other related diseases which indicates that *C. elegans* can be a good predictive screening tool for new potential probiotic strains which are reported to extend the life span exerting beneficial effects in human hosts.

Therefore, the objective of this study was to investigate the antimicrobial activity of probiotic cultures against *Cronobacter sakazakii* and the potential of probiotic culture's supernatant to inhibit the biofilm formed by *C. sakazakii.* The immunomodulatory potential and pathogenic inhibitory potential of probiotic isolates were also confirmed using model system *C. elegans.*

5.2 Material and methods

5.2.1 Strains and culture conditions

- The pathogenic standard strain *C. sakazakii* ATCC 12868 used in the study was grown in tryptone soy broth at 37°C for 12 h [9].
- Probiotic cultures isolated and reported in first objective were grown in MRS media at 37°C for 24 h.
- The wild type strain of *C. elegans* (Bristol N2) was maintained on NGM as described in previous chapter
- *Escherichia coli* OP50, was grown on Luria Bertani (Hi-media Laboratories, Mumbai) agar plates at 37°C [10].

5.2.2 Preparation of cell free supernatant of probiotic isolates

CFS of probiotic bacteria isolated from fermented dairy products and fermented foods [11][12] was prepared using the following protocol:

- The cultures were grown in MRS broth at 37ºC for 48 h in shaking conditions at 100 rpm. After 48 h of the incubation, the broth was centrifuged at 8000 g for 5 min.
- The supernatant was collected in a separate tube and pellet was discarded. Bacterial supernatant was filter sterilized with a syringe filter (0.2 µm) (Chromatographic Specialties Inc., ON, Canada) to get CFS.
- This supernatant was divided into three fractions for each culture i.e. untreated cell free supernatant (CFS), heat treated cell free supernatant (hCFS) (70°C for 20 min) and pH neutralized cell free supernatant (NnCFS) (pH 6.8 with 1 M NaOH). All the treated and untreated CFS was stored in -20°C for further use.

5.2.3 Antimicrobial susceptibility of probiotics against *C. sakazakii*

- Antimicrobial susceptibility of probiotics against *C. sakazakii* was measured using agar well diffusion method. [13]

The indicator strain of *C. sakazakii* was grown in TSB overnight. After incubation, the OD_{600nm} of culture broth of the *C. sakazakii* strain was adjusted to 0.5

A 100 µl of culture broth was spread on Muller Hinton Agar plates with the help of glass spreader and wells were dug in agar plates

A 100 µl of untreated CFS and treated i.e. hCFS and nCFS of probiotic cultures was added in the each well on the plate.

The plates were incubated for 24 h at 37°C and were examined for the zone of inhibition surrounding the wells

The diameter of zones of inhibition was measured and the observations were recorded

5.2.4 Minimum inhibitory concentration (MIC) of CFS

MIC is reported as the lowest concentration of CFS that produces visible inhibition of microbial growth and significant reduction in the viable counts of the bacteria [14].

Preparation of CFS from overnight grown probiotic cultures in MRS broth

↓

A 10 µl of overnight grown *C. sakazakii* and CFS, each, added in the dose-dependent manner (30µl, 40 µl and 50µl) into varying levels of fresh TSB

↓

Total Volume in wells raised to 250 µl by TSB

↓

Incubation of MTP for 24 h at 37°C under static conditions.

↓

Absorbance of cell biomass at 570 nm using UV- spectrophotometer (Multi-Skan Go Microplate reader, Thermo Fischer Scientific, USA).

↓

Determination of MIC as the lowest concentration of CFS that produced complete suppression of visible growth.

↓

40 µl dose of CFS was selected as MIC for further experiments

5.2.5 Inhibition of *C. sakazakii* biofilm formation by probiotic isolates

The effect of CFS on the biofilm inhibition of *C. sakazakii* was determined by standard crystal violet staining assay using microtiter plate (MTP) whereby the inhibition potential was recorded by decrease in absorbance of biofilm associated cells [15].

A 10 µl of overnight grown *C. sakazakii* isolate and 40 µl of CFS was added in 200 µl of fresh TSB medium.

Incubated the MTP under static conditions for 24 h at 37°C.

Washing of non-adherent cells twice with phosphate buffer saline (PBS, pH 7.0).

The surface-adhered/ biofilm cells in the ells were stained with 200 µl of 0.2 % crystal violet solution.

Removal of excess CV solution after 15 min of incubation

Solublization of stained biomass in 250 µl of 33 % (v/v) glacial acetic acid.

Quantification of biofilm associated cell biomass at OD_{570} nm using UV-spectrophotometer using sterile TSB as blank

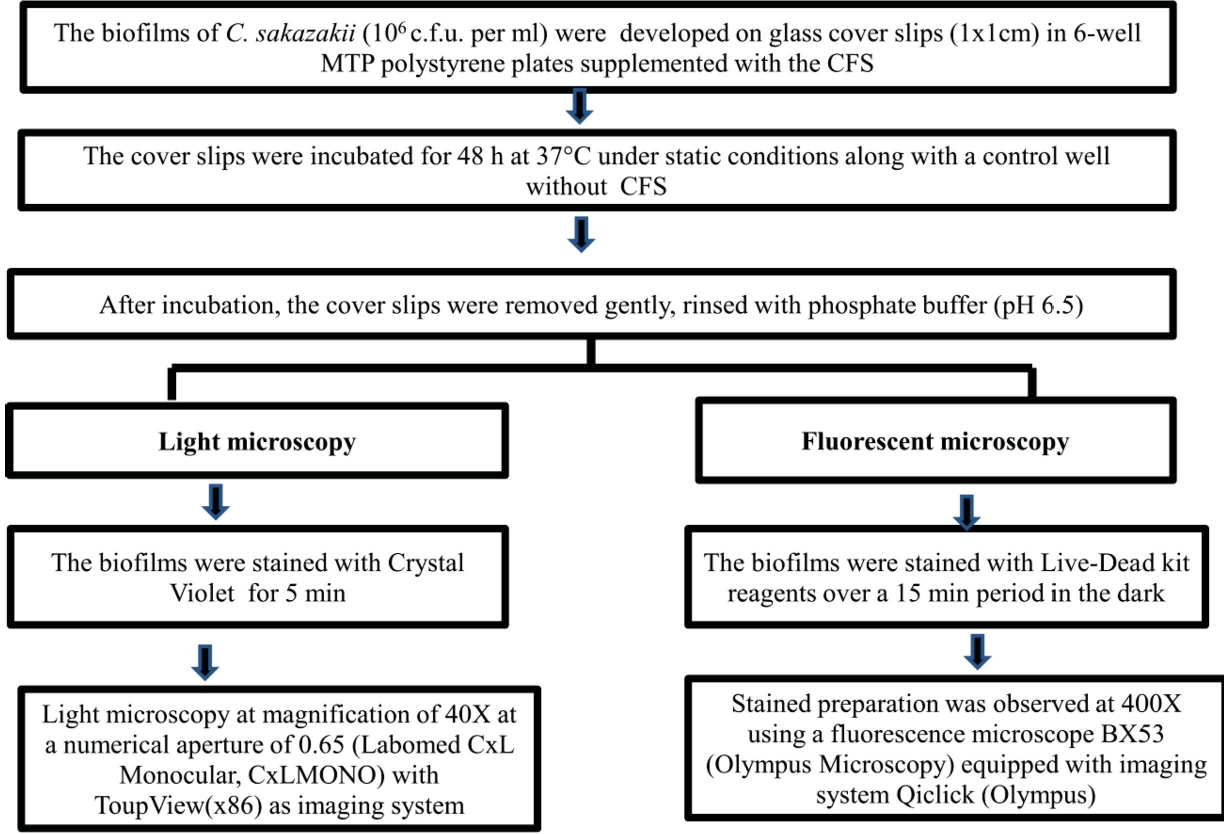
Determination of % of biofilm inhibition
$(controlOD_{570nm}-testOD_{570nm}/controlOD_{570\,nm})100$

5.2.6 Post biofilm inhibition by CFS of probiotic isolates

The post-biofilm inhibition was conducted to check the efficiency of CFS to inhibit the mature biofilms [16].

- In post incubation assay the pathogenic biofilm was allowed to form for different time intervals (12h, 24 h and 48h)
- After the formation of biofilms, it was treated with treated and untreated CFS (40 µl) and incubated for next 24 h.
- The effect of CFS on biofilm biomass was observed after incubation as described in above section.

5.2.7 Microscopic observations of biofilms.



5.2.8 Pathogenic potential of *C. sakazakii*

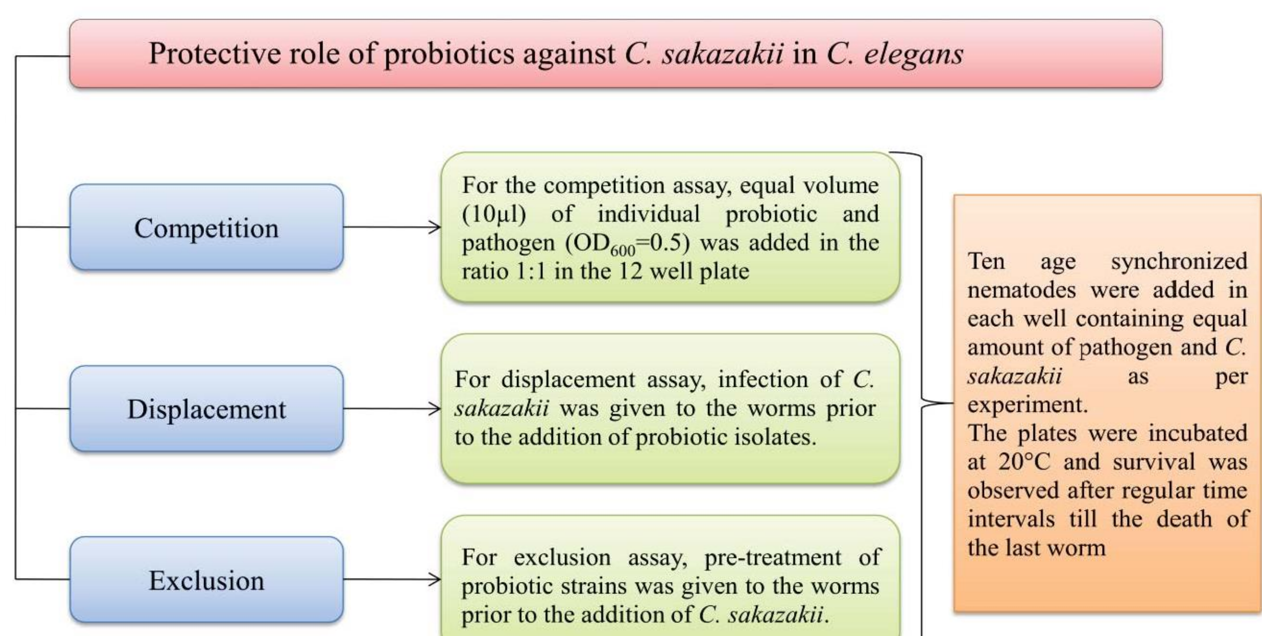
The pathogenic potential of *C.sakazakii* in the nematode was determined using liquid killing assay [18][19]. It should be noted that unless otherwise specified, all the assays were performed under liquid conditions only. The assay was performed as per the protocol described in chapter 4.

5.2.9 Chemotaxis and pharyngeal pumping assay

The physiological changes, pharyngeal pumping and binary choice assays were conducted as described [20] in chapter 4.

5.2.10 Protective effects of probiotics

The probiotic strains were selected based on the antimicrobial activity against *C. sakazakii* and were used for competitive exclusion assays against *C. sakazkii*. The protective role of probiotic isolates and strain-specific abilities against *C. sakazakii* was evaluated in liquid media as follows [21].



5.2.11 Statistical Analysis

All the experiments were performed in triplicates, and the comparison of the strains for their effect on the host was done by ANOVA and Tukey's multiple comparison test ($p<0.05$) by SPSS software.

5.3 Results and Discussion

The present study has reported the protective role of probiotic against infection by *C. sakazakii* using *in vitro* tests and *in vivo C. elegans* model system. The present study investigates the potential of probiotic bacteria isolated from traditional fermented foods against infection and colonization of *C. sakazakii* using *in vitro* and *in vivo* studies. The *in vitro* assays were used to determine the antimicrobial activities of different probiotics against *C. sakazakii*, the selected strains were used to determine their potential to eradicate the biofilm formation potential of *C. sakazakii*. Further the four probiotic strains selected through *in vitro* studies were used to determine the competitive exclusion activities against *C. sakazakii* in established model system *C. elegans*. The protective role of probiotic supplementation was determined in terms of survival rate of the worms after infection as well as physiological and morphological changes in worm due to infection. Protective role of probiotic isolates was also checked by competitive exclusion of pathogen in different assays.

5.3.1 Antimicrobial activities of probiotics

The results of antimicrobial activities of fifteen probiotic isolates against *C. sakazakii* as determined by different fractions of cell free supernatant of lactic cultures are presented in Table 5.1. The CFS and hCFS of all the strains possess antimicrobial activities against *C. sakazakii* strain with a diameter of zone of inhibition ranging from 14-24 mm. In general, the inhibition level was slightly lower with hCFS compared to untreated CFS whereas no zone of inhibition was observed in the wells containing pH neutralized CFS. The highest inhibitory activity of CFS was registered for *L. gastricus* BTM7, *L. plantarum* K90 and lowest was observed in *L. fermentum* K100, *L. fermentum* K75 and *W. confuse* against *C. sakazakii*. Varying degree of the antimicrobial activity of CFS against *C. sakazakii* was observed using different preparation of CFS. To confirm the presence of non-proteinaceous components in exhibiting antimicrobial

activity, the CFS was subjected to heat treatment and neutralized for acidity. As there was no activity in pH neutralized CFS, it indicates that the antimicrobial component is acidic in nature [22]. The results clearly indicated the role of organic acids the antagonistic activity of probiotics against *C. sakazakii*. The increased production of lactic acid through fermentation (which was 48 h in the present study) reduces pH (up to 4.0) of the media, which is reported to inhibit the growth of most food-borne pathogens. This antimicrobial activity is also reported due to the undissociated forms of the acid and its capacity to reduce the intracellular pH. The reduced pH led to inhibition of vital cellular functions. Although the role of other antimicrobial components such as metabolic products (short-chain acids, hydrogen peroxide), competition for nutrients and production of inhibitory substances with protein (bacteriocins or bacteriocin like compounds) [23][24], also found that organic acids, for example, lactic acid likewise works as a membrane permeabilizing of the Gram-negative bacterial external membrane and may go about as a potentiator of the impacts of other antimicrobial substances. The outcomes demonstrated profoundly critical antibacterial effects against these pathogens, particularly for *L. paracasei* CD4 and *L. gastricus* BTM7. This antibacterial activity could be due to production of antimicrobial metabolites such as acids and others and bacteriocins The antimicrobial activities of *L. paracasei* have been reported previously against diarrheal pathogens and enteric Staphylococcal diseases
[25] through production of bacteriocins like substances.

Table 5.1: ***In vitro*** **antimicrobial activities of probiotic bacteria against** ***C. sakazakii*** **(ATCC 12868)**

	Zone of inhibition (mm)		
Isolates	**(CFS**	**hCFS**	**nCFS**
L. paracasei CD4	20^{bd}±1.1	20^{ce}±1.1	-
L. gastricus BTM7	24^{e}±1.1	22^{e}±1.1	-
Brev.. aydinogluensis BTM9	18^{ac}±0	18^{be}±1.1	-
Brev. thermoruber CD13	18^{bd}±1.1	16^{bc}±1.1	-
*Enterococcus sp.*GTM14	18^{bd}±1.1	18^{be}±1.1	-
Brevi. thermoruber HM29	18^{a}±1.1	20^{de}±0	-
Brevi. thermoruber HM34	20^{bd}±1.1	18^{be}±1.1	-
W. confuse CD1	16^{ab}±1.1	16^{bc}±1.1	-
L. rhamunosus GG	20^{ce}±1.1	18^{be}±1.1	-
L. fermentum K75	16^{a}±1.1	16^{ab}±0	-
L. fermentum K78	18^{ab}±1.1	20^{ce}±1.1	-
L. plantarum K84	20^{bd}±1.1	18^{bd}±1.1	-
L. plantarum K90	22^{de}±0	22^{e}±1.1	-
Pedio. acidilactici K94	18^{bd}±1.1	18^{be}±1.1	-
Pedio. acidilactici K98	20^{bd}±1.1	18^{bd}±0	-
L. fermentum K100	16^{a}±1.1	14^{a}±1.1	-

"a-e Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)"

5.3.2 Minimum inhibitory concentration (MIC) of CFS

The MIC assay revealed different susceptibilities of C.sakazakii to the different fractions of CFS of probiotic strains, and exhibited potent inhibitory effect as represented as MIC values different doses (30-50 µl) of CFS were used to determine the MIC against *C. sakazakii*. Dose dependent decrease in growth of indicator strain was observed whereby complete inhibition was observed with a dose of 50 µl whereas lower inhibition was measured with the dose of 30 µl and 40 µl ($p<0.05$). Based on the results, for further studies, a moderate dose of 40 µl was used. In our present study we have found that the cell free supernatant of probiotic bacteria was able to inhibit the biofilm formed by food pathogen *C. sakazakii* (Fig. 5.1). In a study done by [26], the authors reported that Lc. lactis CLFP 101 had reduced the adhesion of pathogens in fish, whereas *L. plantarum* CLFP 238 reduced the adhesion of Aeromonas hydrophila and *A. salmonicida*. In their study *L. fermentum* CLFP 242 and mixture of the three LAB strains reduced the adhesion of all fish pathogens to intestinal mucus, thus showing the potential of lactic acid bacteria to reduce pathogenic biofilm in the intestine.

5.3.3 Biofilm inhibitory activities of CFS

The effects of CFS of selected probiotic strains on the biofilm formation and disruption of mature biofilms by *C. sakazakii* were analyzed by using standard crystal violet assay. The co-incubation experiment where the cultures were tested for their ability to inhibit the biofilm formation using all fractions of CFS (CFS, hCFS and nCFS) indicated that the untreated and heat treated CFS had maximum inhibitory activity against formation of biofilm whereas the neutralized supernatant was the weakest among all tested fractions. The maximum inhibition in wells treated with untreated CFS of *L. paracasei* CD4 and *L. gastricus* BTM7 and the lowest inhibition was observed for *L. fermentum* K75 (48%). Further the ability of the probiotic strains were tested to eradicate the mature biofilm of *C. sakazakii* using post incubation assay, where the biofilm formed after 12, 24 and 48 h was treated with different fractions of CFS (treated and untreated) and results were expressed in % biofilm inhibition potential (Table 5.3). Similar to co-incubation assay, the untreated CFS resulted in maximum biofilm eradication ability followed by hCFS and minimum inhibitory activity with pH neutralized CFS.

Table 5.2: Minimum inhibitory concentration (MIC) of cell free supernatant of probiotic cultures against *C. sakazakii*

Isolates	CFS (30µl)	CFS (40µl)	CFS (50µl)
L. paracasei CD4	0.06^{cd}±0.02	0.02^{ab}±0.01	0.01^{a}±0.003
L. gastricus BTM7	0.08^{ef}±0.11	0.04^{cd}±0.0	0.02^{ab}±0.0
Brev. aydinogluensis BTM9	0.03^{a}±0.02	0.012^{ab}±0	0.01^{a}±0.0
Brev. thermoruber CD13	0.087^{ef}±0.03	0.02^{ab}±0.001	0.01^{a}±0.0
Enterococcus sp. GTM14	0.08^{ef}±0.12	0.01^{a}±0.003	0.01^{a}±0.0
Brevi. thermoruber HM29	0.05^{bc}±0.001	0.01^{a}±0.0	0.01^{a}±0.0
Brevi. thermoruber HM34	0.08^{ef}±0.03	0.01^{a}±0.0	0.01^{a}±0.0
W. confuse CD1	0.04^{ab}±0.002	0.014^{a}±0.00	0.01^{a}±0.0
L. rhamunosus GG	0.04^{ab}±0.01	0.03^{bc}±0.001	0.03^{b}±0.0
L. fermentum K75	0.095^{f}±0.004	0.07^{e}±0.04	0.06^{c}±0.06
L. fermentum K78	0.07^{de}±0.002	0.05^{d}±0.17	0.01^{a}±0.0
L. plantarum K84	0.089^{ef}±0.001	0.050^{d}±0.10	0.01^{a}±0.0
L. plantarum K90	0.09^{f}±0.04	0.04^{cd}±0.03	0.01^{a}±0.001
Pedio. acidilactici K94	0.06^{cd}±0.02	0.01^{a}±0.0	0.015^{a}±0.00
Pedio. acidilactici K98	0.05^{bc}±0.001	0.01^{a}±0	0.01^{a}±0.003
L. fermentum K100	0.03^{a}±0.001	0.02^{ab}±0	0.02^{ab}±0.003

O.D. of positive control = 0.96±.002 at 600nm.

"a-f Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. ($p<0.05$)"

Table 5.3 Biofilm inhibitory assay showing percent inhibition (Coincubation) (24 hour)

Isolates	CFS	Hcfs	nCFS
L. paracasei CD4	92.92^{g}± 0.16	79.59^{f}±0.05	29.45^{g}±0.08
L. gastricus BTM7	92.05^{g}±0.0	85.56^{gh}±0.06	32.26^{g}±0.04
Brevi. aydinogluensis BTM9	90.15^{eg}±0.01	86.63^{gh}±0.06	15.31^{e}±0.07
Brevi. thermoruber CD13	88.24^{dg}±0.01	89.53^{h}±0.0	22.26^{f}±0.02
Enterococcus sp. GTM14	74.69^{c}±0.17	82.63^{fg}±0.06	23.59^{f}±0.05
Brevi. thermoruber HM29	87.87^{dg}±0.04	86.67^{gh}±0.03	7.56^{bc}±0.11
Brevi. thermoruber HM34	85.77^{df}±0.1	86.96^{gh}±0.07	14.60^{de}±0.04
W. confuse CD1	91.30^{fg}±0.0	81.11^{fg}±0.0	8.39±^{bc}0.06
L. rhamunosus GG	78.32^{c}±0.08	54.54^{c}±0.17	10.38^{cd}±0.06
L. fermentum K75	48.95^{a}±0.21	34.56^{b}±0.01	4.59^{ab}±0.07
L. fermentum K78	63.63^{b}±0.13	24.67^{a}±0.31	15.48^{e}±0.04
L. plantarum K84	85.01^{de}±0.0	86.91^{gh}±0.0	6.99^{bc}±0.04
L. plantarum K90	87.74^{dg}±0.01	85.21^{fh}±0.01	17.58^{e}±0.04
P. acidilactici K94	84.31^{d}±0.0	85.81^{fh}±0.01	34.56^{g}±0.07
P. acidilactici K98	65.13^{b}±0.06	65.43^{d}±0.02	2.49^{a}±0.01
L. fermentum K100	67.15^{e}±0.04	72.50^{e}±0.03	10.18^{cd}±0.07

Concentration of CFS, hCFS and nCFS=40 µl

“a-g Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey’s – post-hoc range test between replications. ($p<0.05$)”

Effects of CFS on biofilms of *C. sakazakii*

The stained microscopic observations revealed the clear difference between the treated and the control biofilm of *C. sakazakii* (Fig.5.2 and Fig.5.3). The slides without any treatment were observed to have intact, uniform, consistent and well-developed biofilm whereas the slides treated with CFS were observed to have scattered biofilm. The fluorescent microscope images also indicated a major disruption in the architecture of biofilms of *C. sakazakii*.

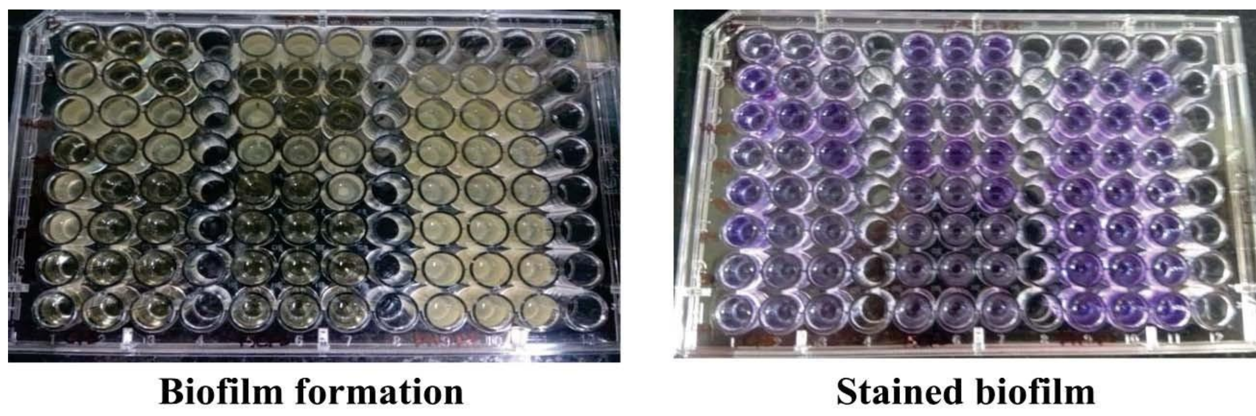


Figure 5.1: Plate assay showing biofilm inhibition

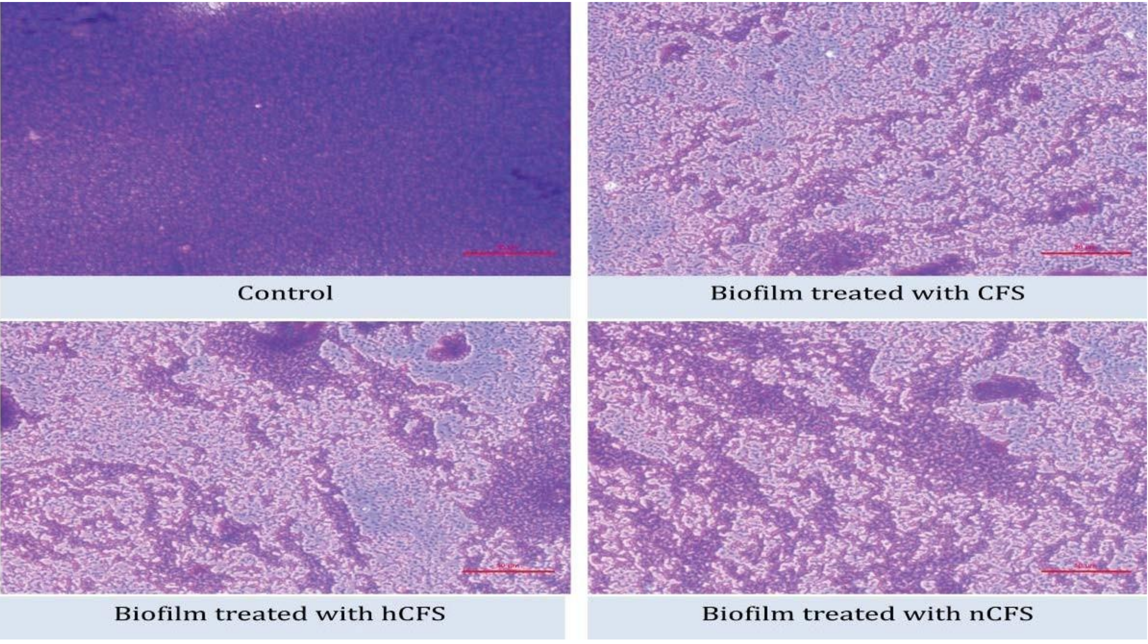
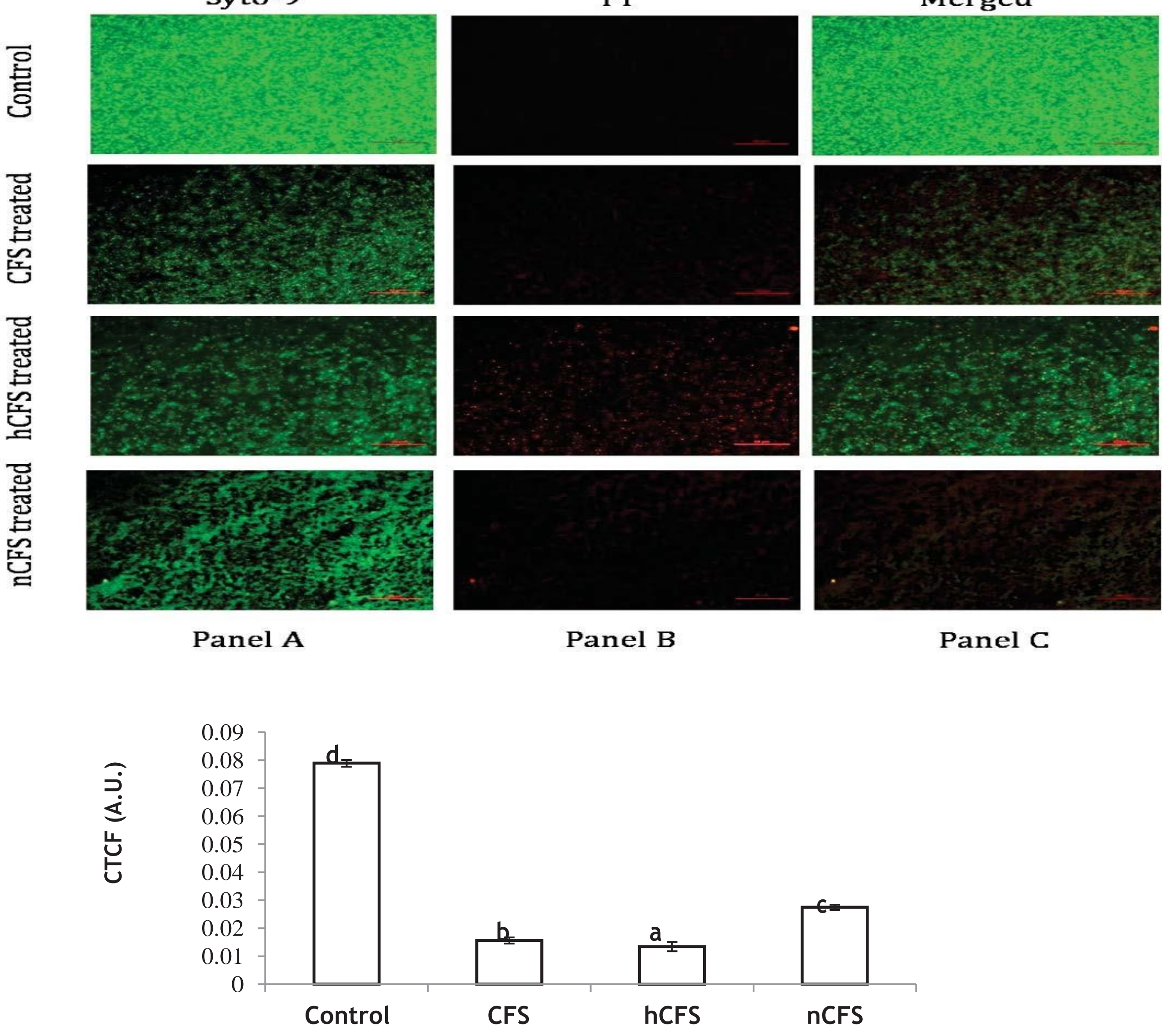


Figure 5.2: Effects of different fractions of CFS on the biofilms of *Cronobacter sakazakii* stained with crystal violet.



“a-d Means in the column with different superscript letter are significantly different as measured by 2 sided Tukey’s – post-hoc range test between replications. ($p<0.05$)”

Figure 5.3: Effects of different fractions of CFS on biofilms of *C. sakazakii* grown determined by fluorescent microscopy using LIVE/DEAD Bac Light bacterial viability kit Panel A (left) is the image obtained from the green channel (Syto 9 stained), panel B (center) from the red channel (Propidium iodide stained) and panel C (right) is a merged image. Corrected total cell fluorescence (CTCF) ratio of different groups.

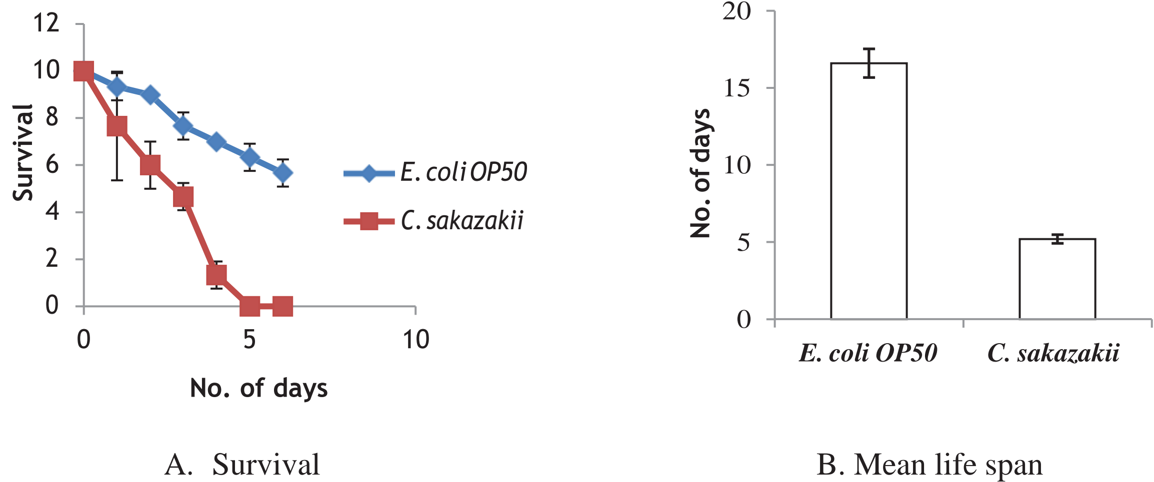
5.3.4 Protective effect of probiotics

Protective role of probiotic cultures against *C. sakazakii* infection was observed in three different CE assays. As observed from competitive assay where probiotic and pathogen were administered in *C. elegans* in equal proportions (1:1), as well as the post infection treatment with probiotic (displacement assay), the mean life span of the worm was extended to 5-6 days as compared to control without any probiotic intervention. Whereas, in exclusion assay, the preconditioning with probiotic cultures resulted in increased mean life span of the nematode to 12-13 days. As probiotics are known for their antimicrobial properties *in vivo* and *in vitro* therefore the CE assays were performed to observe the effect of intervention of probiotics on killing or eradication of the pathogen colonization. The results indicated that among all the three CE assays, inhibition, exclusion and displacement assays, the displacement was observed to be the best strategy to limit the infection of pathogen. The pre-colonization by probiotic bacteria in the intestine of worm didn't allow pathogens to adhere to gut of nematodes and hence prevented it from infection by *C. sakazakii*. Similar results were reported in *C. elegans* whereby preconditioning the worm with *Lactobacillus acidophilus* Strain NCFM resulted in less susceptibility to E. faecalis infection in the liquid killing assay and enhanced the viability of nematodes on solid agar assay [27].

5.3.5 Pathogenic potential of *C. sakazakii*

The pathogenic potential of *C. sakazakii* was determined by feeding *C. elegans* with *C. sakazakii* to check the survivability of the worms. As shown in Fig. 5.4 and 5.5, the wild type N2 *C. elegans* died faster when fed with *C. sakazakii* as compared to control *E. coli* OP50 (normal food source) under liquid conditions with 50% survival after 3 days of feeding with a total mean life span time of 5 days as compared to control of 16 days. The *C. sakazakii* exposed worms had shown normal behavior for first 5 to 10 h as equivalent to standard feeding of *E. coli* OP50. However, after 10 h the worms started showing several impairments such as paralyses, slow movement in media, gametogenesis, poor egg formation, poor vulval growth, internal hatching, distorted intestine and pharynx as shown in Fig.5.6. Feeding of nematode with *C. sakazakii*

affected the pumping rate of pharynx when compared with control (Fig. 4.3b). As the life span of the infected worms were recorded to be 5 to 6 days, the pumping rate dropped down with infection time and finally led to the death of the nematode. In chemotaxis assay, *C. elegans* didn't show pathogen avoidance behavior. Choice index was recorded to be 0 for both test and control plates which represented the equal distribution of worms towards both the zones after 4 hr of incubation. After 24 h of incubation the pattern recorded have shown the same path formed by worms both the sides of zones as shown in the Fig. 5.7. *C. sakazakii* is a Gram-negative opportunistic pathogen associated with invasive infections such as meningitis, bacteraemia and necrotizing enterocolitis in adults and neonates [28]. The high fatality rate of infants has been epidemiologically-related to the consumption of contaminated powdered infant formula (PIF) [29]. "The pathogen is reported to possess few putative virulence markers such as production of proteolytic enzymes, outer membrane proteins, iron acquisition gene systems, however, outer membrane proteins (*OmpA* and *OmpX*) are one of the best characterized virulence factors with a role in the adherence and invasion of *C. sakazakii* with animal cell models." The pathogenic potential of *C. sakazakii* in *C. elegans* has already reported before where similar deformations after internalization of the pathogen lead to killing of the worm [30]. The study also reported that the killing was not due to any release of toxigenic factor in the spend medium. The binary choice index revealed that *C. sakazakii* affects the pharyngeal pumping rate of *C. elegans* along with its olfactory response of nematode towards the pathogen.



A. Survival B. Mean life span

Figure 5.4: Survival curve showing survival and mean life span of *C. elegans* fed with *C. sakazakii*

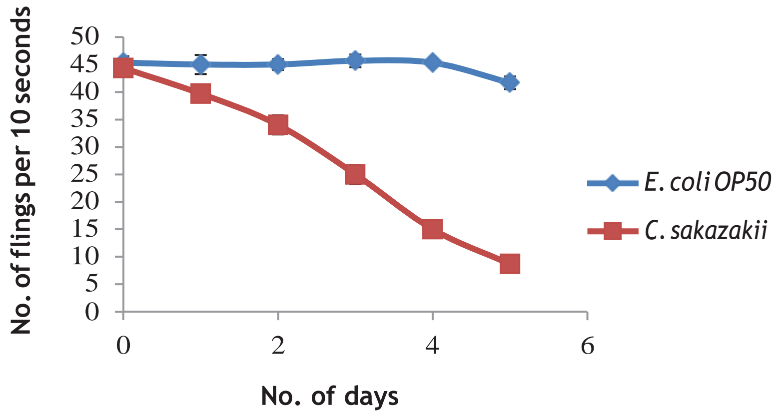


Figure 5.5: Pharyngeal pumping rate of the terminal bulb of pharynx in control *E. coli* OP50 and in *C. sakazakii* infected worm recorded as the number of flings in 10 consecutive seconds for six days

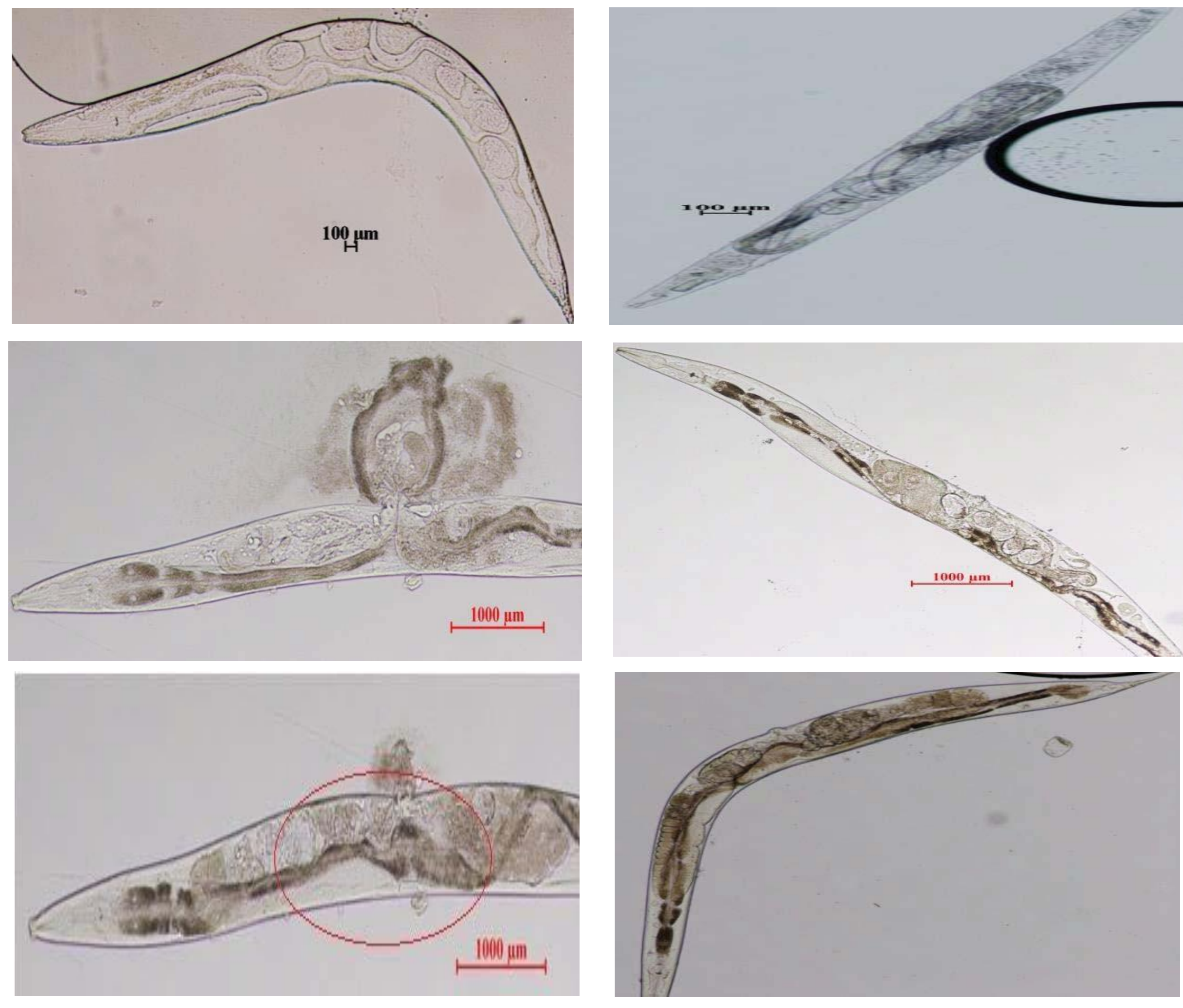
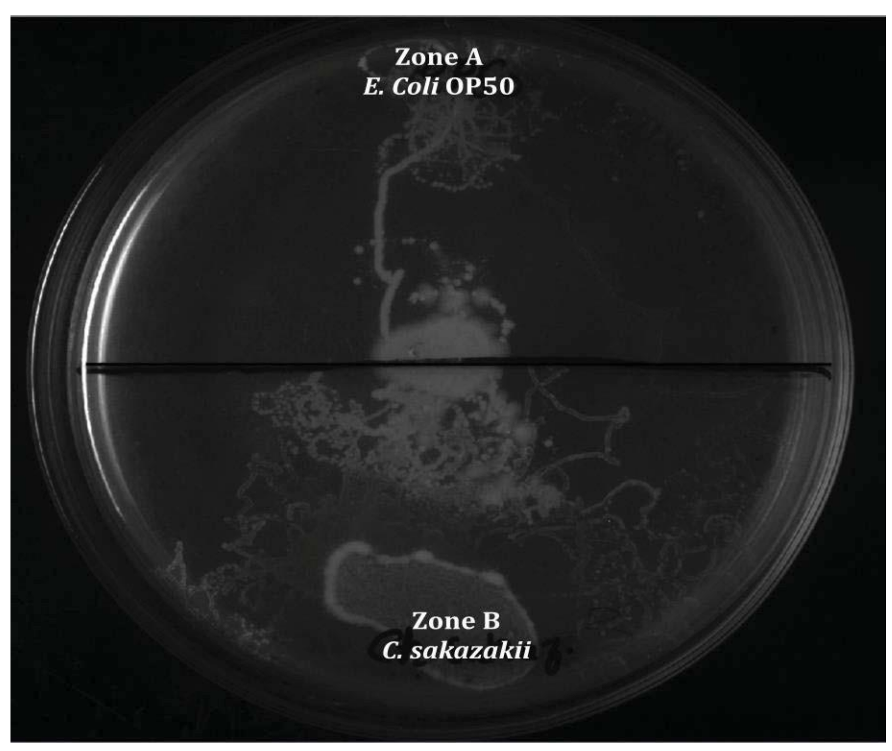


Figure 5.6: Images showing the changes occurred in physiology and morphology of *C. elegans* fed with *C. sakazakii*. Internal hatching, bag of worms, birth defects, poor growth of worms, poor vulval region, swollen intestine and ruptured terminal bulb was observed.



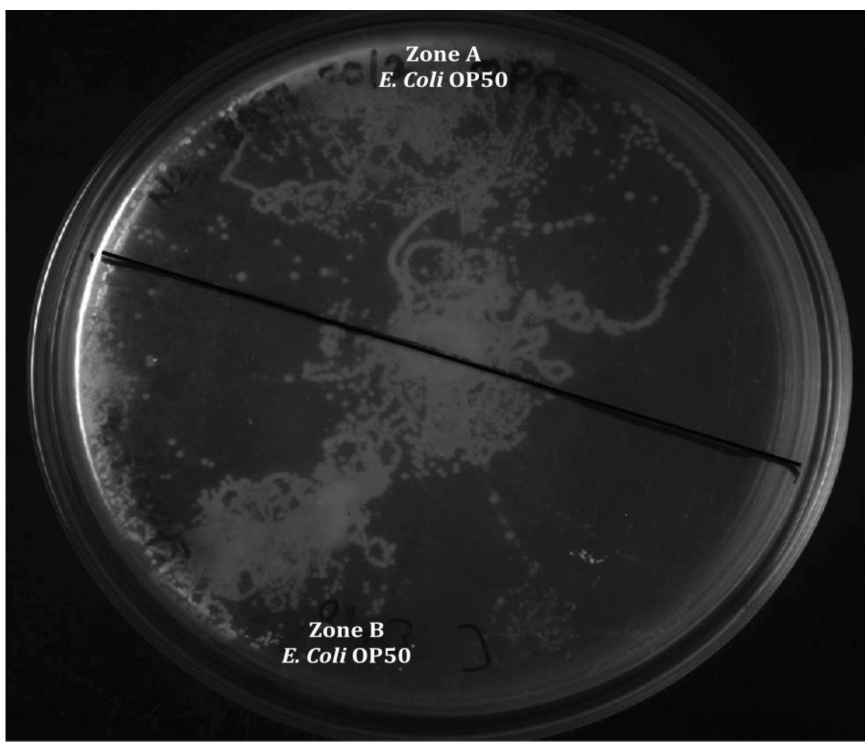


Figure 5.7: Binary choice assay for C.sakazakii and E.coli OP50 in *C. elegans*

5.3.6 Protective role of probiotic against *C. sakazakii* in *C. elegans*

Protective role of probiotic cultures against *C. sakazakii* infection was observed in three different CE assays. As observed from competitive assay where probiotic and pathogen were administered in *C. elegans* in equal proportions (1:1), as well as the post infection treatment with probiotic (displacement assay), the mean life span of the worm was extended to 5-6 days as compared to control without any probiotic intervention (Fig. 5.8) and also in displacement assay where there was also increase of 5-6 days was observed in lie span (Fig. 5.9). Whereas, in exclusion assay, the preconditioning with probiotic cultures resulted in increased mean life span of the nematode to 12-13 days (Fig 5.10). The conclusion of this experiment is that the preconditioning of worms with probiotic isolates prior to infection helped in longevity and reduces the chance of infection by *C. sakazakii* in the worm. Thus, probiotics have enhanced the life span by colonizing and competitively inhibiting the pathogen and hence protecting the worms from infection.

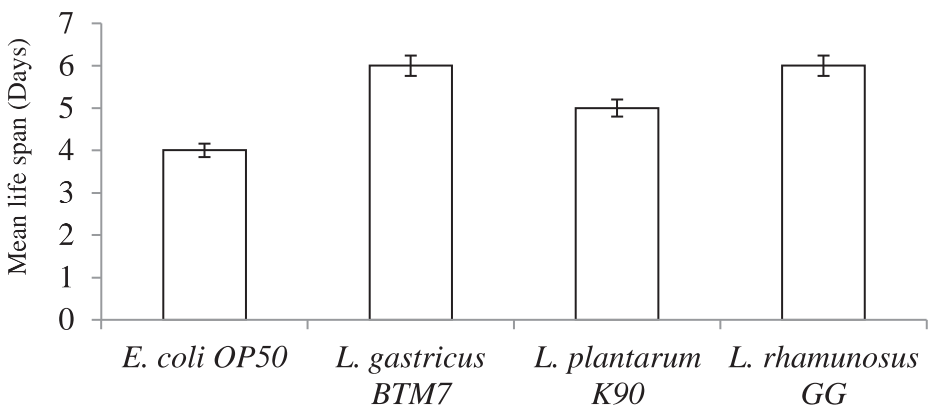


Figure 5.8: Competition assay

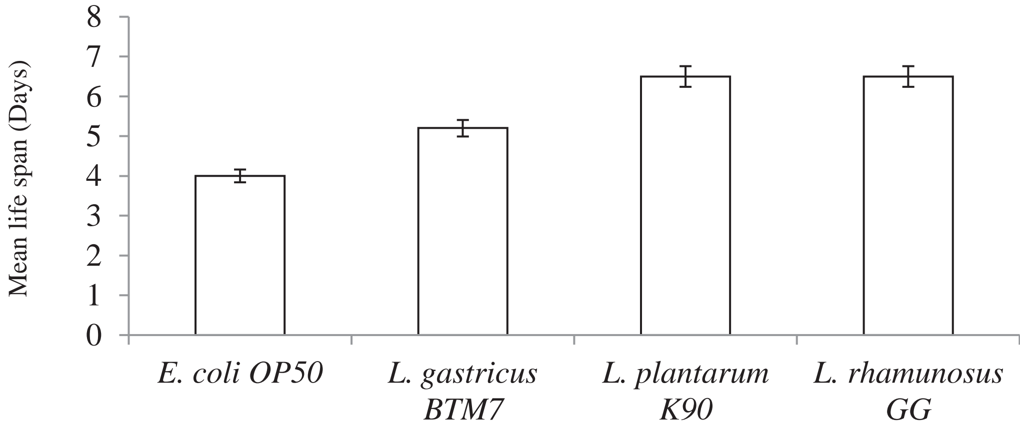


Figure 5.9: Displacement assay

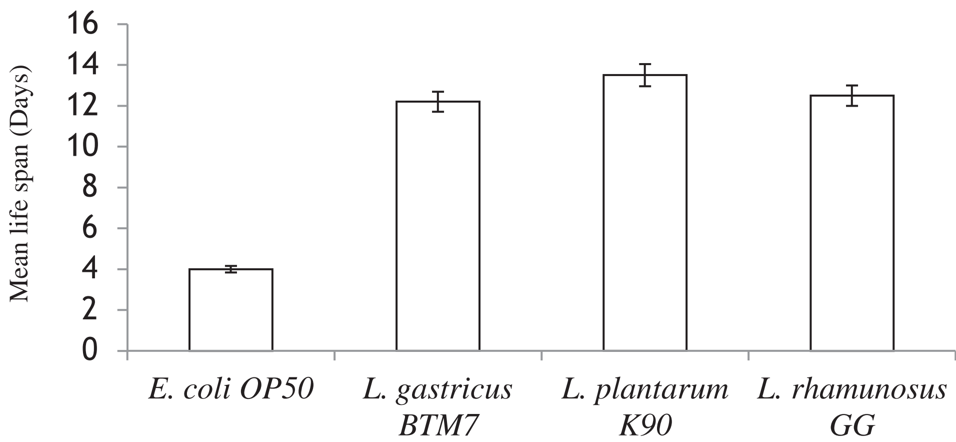


Figure 5.10: Exclusion assay

5.4 Conclusion

Summary of the study have concluded the probiotic potentials of probiotic isolates isolated from fermented food products of Himachal Pradesh. The cell free supernatant from probiotic isolates was found to inhibit the growth of *C. sakazakii* on agar well plates and the CFS was able to inhibit the biofilm formation of *C. sakazakii in vitro*. Probiotic isolates were able to introduce longevity in nematode *C. elegans* and was also able to inhibit the growth of pathogens inside the worms by competitive exclusion. The studies have reported that probiotics are able to upregulate several key genes involved in ageing process in *C. elegans*. Thus, the probiotic isolates are having potential against *C. sakazakii* pathogenesis.

Chapter 6

SUMMARY AND FUTURE SCOPE

SUMMARY AND FUTURE SCOPE

The present research work entitled "Characterization of probiotic cultures from fermented foods of Himachal Pradesh and their efficacy against colonization by *Cronobacter sakazakii* in *Caenorhabditis elegans* model system" was carried out with an aim to isolate and identify new probiotic strains from traditional fermented food products of Himachal Pradesh and to chec their efficacy against infection of *C. sakazkii* in *Caenorhabditis elegans* model. The major findings reported in this thesis are summarized below:

The study targeted milk and cereal based food products for the isoaltion of probiotic lactic cultures. For this, a total of 150 samples (fermented cereal-based dough and fermented dairy samples) were collected from different places and districts of Himachal Pradesh. The primary isolation was done on MRS agar where from these samples we obtained 101 distinct isolates based on their colony characteristics. The colonies were opaque, white and creamish colonies. All these isolates were identified as Gram-positive rods or cocci, facultative anaerobes and catalase negative. Subjecting to safety attributes depicting hemolytic activity on sheep blood agar plates, out of 101, 51 isolates were found to be non-hemolytic as these isolates did not produce any zone of clearance on blood agar plates. Rest of the isolates resulted in hemolytic reaction. The non-hemolytic isolates were further screened for their survival under GI tract conditions which is a prerequiste for a probiotic candidate.

Out of 51 isolates 15 isolates were able to pass through the acidic pH of stomach and bile salt conditions of intestine. These 15 isolates were further investigated for simulated GI tract conditions and further checked for *in vitro* and *in vivo* probiotic attributes as per joint protocols given by "Department of Biotechnology (DBT), Govt. of India and Indian Council of Medical Research (ICMR), Govt of India".

These fifteen isolates were subjected for identification using standard biochemical assays and molecular apporaches targeting 16SrRNA region. These isolates were identified using the 16S rDNA region: *Brevibacillus thermoruber* (three strains), *Lactobacillus gastricus, Brevibacillus aydinogluensis, Enterococcus sp., L. paracasei, Weisella confuse*, *Lactobacillus fermentum* (three strains), *L. plantarum* (two strains), and *Pediococcus acidilactici* (two strains).

The most promising strains (14 strains) were identified after testing their resistance, by using subtractive screening, to digestive gastrointestinal system barriers (acid and digestive fluid salts). The isolates were screened for their survial at highly acidic conditions (pH 2.0), digestive fluid salts (1%), and pancreatin (1mg/L) for different time intervals. The survival was determined using viable cell count method.

The cultures were also tested for their adhesion potential using in vitro methods for cell autoaggregation, cell surface hydrophobicity, and adhesion to Caco-2 cell line and mucin. All the isolates were able to adhere to Caco-2 cell lines and resulted in varying degree of autoaggregation and hydrophobicity to n-hexane. Among the tested strains, the maximal tolerance to a simulated gastric environment was observed in *L. paracasei* CD4 and *L. gastricus* BTM7 with higher scores in adherence studies.

After confirming the probiotic activities of these strains, the investigation was done to analyse their benefits towards functioning of *C. elegans (Caenorhabditis elegans) as* in vivo model. The protective effect of probiotics was assessed by increase in life expectancy of worms by colonizing inside the bowel. All the fifteen cultures were administered individually to age synchronized worms and their impact on mean life expectancy of *Caenorhabditis elegans* was calculated. The impact on pharyngeal pumping, normal reproduction and chemotactic conduct (binary choice assay) was conjointly determined. The colonization potential of these probiotics was also determined at different time intervals after the administration of the probiotics to the worms using microscopic fluorescent observations and population of microbes. The examination of survival was done by percent survival of the worm which demonstrated that the probiotic cultures enhances the survival possibility when compared with the control strain *E.coli* OP50. Feeding probiotics does not have any consequence on physiology of the worm like the reproduction behavior and pharyngeal pumping. The strain displays superior colonization and adherence within the worm's gut and expands the life expectancy upto five days when compared with control *E. coli* OP50. However, the extension of life span of the worms was strain specific with maximum expectancy was observed for *L. paracasei* CD4. The worms also preferred probiotic cultures as their foods in comparison to standard *E. coli* OP50.

The in vivo studies in *C. elegans* established the fact these probiotic cultures are beneficial for the worm therefore, further study was targeted on the antimicrobial potential of these strains

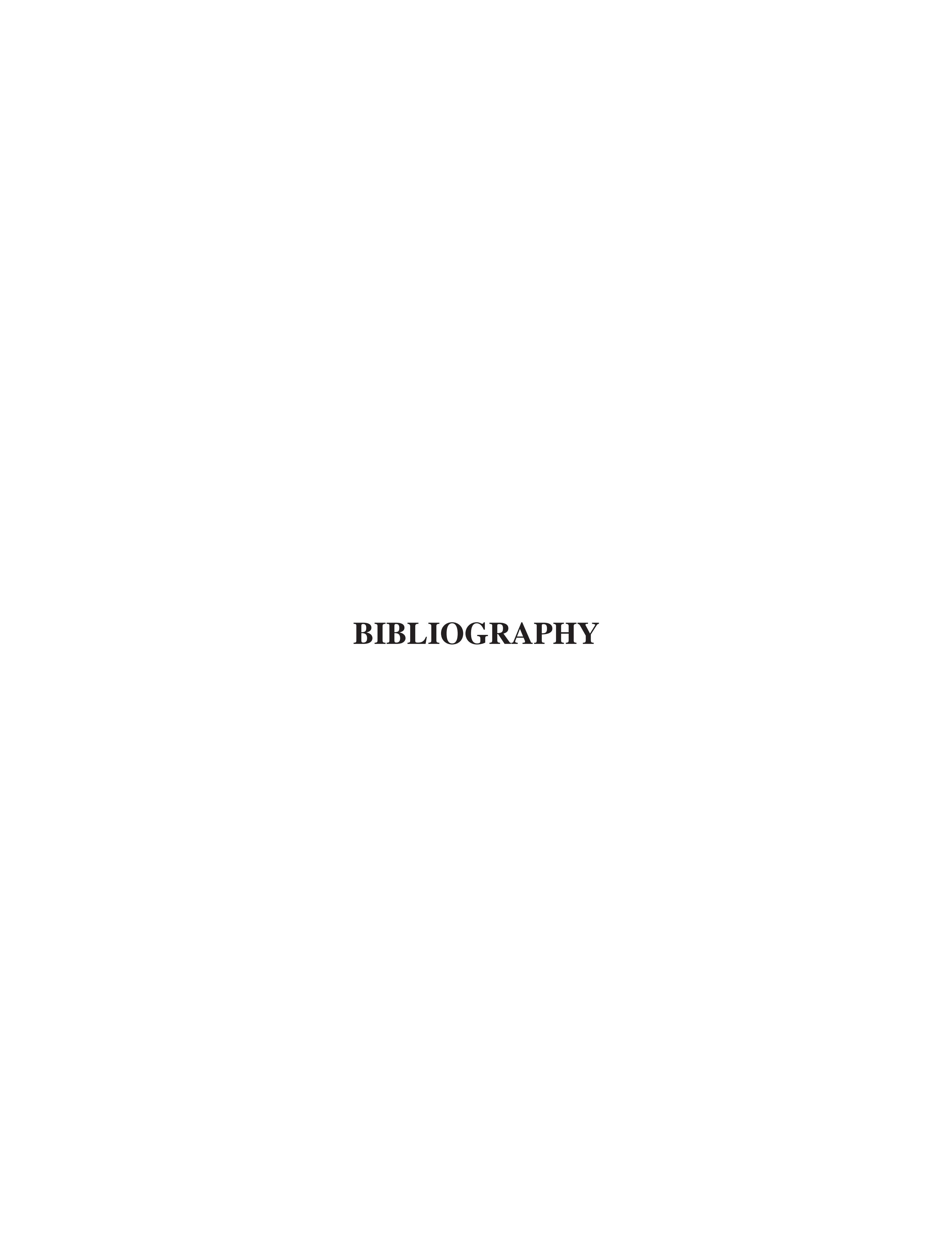
using *in vitro* as well as *in vivo*. The challenge studies were performed in *Caenorhabditis elegans* infected with *C. sakazakii* strain. The antimicrobial activity was assessed by agar well diffusion assay and anti-biofilm forming potential was determined by standard Crystal Violet microtiter plate assay. The probiotic cultures exhibited varying degree of antimicrobial activities against the *C. sakazakii* by different preparations of CFS (heat inactivated and neutralized). The cell free supernatant (CFS) of *L. gastricus* BTM7 and *L. plantarum* K90 exhibited maximum antimicrobial activity against *C. sakazakii*. No inhibitory activities were observed in neutralized CFS indicating the potential role of organic acids or other metabolites displaying antimicrobial activity. A 40 µl concentration of CFS was found to possess highest biofilm inhibition potential as indicated by light and fluorescent microscopic images.

The pathogenic potential of *C. sakazakii* was" determined using liquid media in *C. elegans*, where the *C. sakazkii* strain resulted in complete killing of the worm in 5 days. When compared to standard food of *E. coli* OP50 with a mean life span (MLS) of 16 days. The pathogen also resulted in impaired pharynx, distorted intestine, poor valval growth and internal hatching of the eggs in the worms."

The effects of probiotics against the infection of *C. sakazakii* in *C. elegans* were determined via competitive exclusion assays. Three assays were conducted in CE: competition, displacement and the significant increase in MLS of two to three days was observed for the worms in competition and displacement assays. A pretreatment with probiotic isolates "was found to result in better protection of the worm against infection with *C. sakazakii* by extending the life of the worm by five to six days indicating that preconditioning with probiotic lactic acid bacteria can be taken as an effective measure to overcome the invasion and colonization by the pathogens".

In conclusion, the results obtained in this study contribute to the knowledge of the diversity of LAB microbiota present in traditional fermented foods and dairy products of Himachal Pradesh. The dairy and cereal based fermented foods are an important reservoir of beneficial bacterial cultures mostly belonging to the group of lactic acid bacteria. Not all lactic acid bacteria present in these foods could serve as probiotics as most of the isolates were not able to tolerate the simulated gastrointestinal fluid conditions. The strain specific activities were obtained for adhesion potential of the strains in vitro as well as in vivo. These probiotics could be used as pro-phylactic measures to prevent infections with *C. sakazakii.* The study also

established the fact that the *C. elegans* can be used as an efficient *in vivo* model to screen the probiotics for their effect on life span and intestinal colonization.



BIBLIOGRAPHY

References (Chapter 1)

[1] R. W. Hutkins, “Microbiology and technology of fermented foods,” *John Wiley & Sons*, vol. *22, 2008.*

[2] G. Giraffa, “Studying the dynamics of microbial populations during food fermentation,” *FEMS Microbiology Reviews*, vol. *28*(2), pp. 251-260, 2004.

[3] K. Abegaz, F. Beyene, T. Langsrud J. A. Narvhus, “Indigenous processing methods and raw materials of borde, an Ethiopian traditional fermented beverage,” *Journal of Food Technology in Africa*, vol. *7*(2), pp. 59-64, 2002.

[4] R. W. Hutkins, “Microbiology and Technology of Fermented Foods, 2nd Edition,” *Hoboken, NJ: Wiley*, 2018.

[5] M. L. Marco and B. L. Golomb, “Fermented foods, Lactobacillus, and health,” *Microbe,* vol. *11*, pp. 349–354, 2016.

[6] H. Y. Wang, L. W. Qi, C. Z. Wang and P. Li, “Bioactivity enhancement of herbal supplements by intestinal microbiota focusing on ginsenosides,” *The American journal of Chinese medicine*, vol. *39*(6), pp. 1103-1115, 2011.

[7] J. H. Lee, J. H. Lee and J. S. Jin, “Fermentation of traditional medicine: present and future,” *Oriental Pharmacy and Experimental Medicine*, vol. *12*(3), pp. 163-165, 2012.

[8] M. J. Slashinski, S. A. McCurdy, L. S. Achenbaum, S. N. Whitney and A. L. McGuire, “Snake-oil, quack medicine, and industrially cultured organisms: biovalue and the commercialization of human microbiome research,” *BMC medical ethics*, vol. *13*(1), p. 28, 2012.

[9] N. Thakur, S. Bhalla T. C. Bhalla, “Microbiological and biochemical characterization of experimentally produced Sura-a traditional fermented millet based alcoholic beverage of Kullu District of Himachal Pradesh, India,” *International Journal of Food and Fermentation Technology,* vol. *5*(2), p. 129, 2015.

[10] N. Thakur, P. E. Saris and T. C. Bhalla, “Microorganisms associated with amylolytic starters and traditional fermented alcoholic beverages of North Western Himalayas in India,” *Food Bioscience*, vol. *11*, pp. 92-96, 2015.

[11] V. K. Joshi, A. Kumar and N. S. Thakur, "Technology of preparation and consumption pattern of traditional alcoholic beverage 'Sur'of Himachal Pradesh," *Int J Food and Ferment Technol.*, vol. *5*(1), pp. 75-82, 2015.

[12] K. J. Heller, "Probiotic bacteria in fermented foods: product characteristics and starter organisms," *The American journal of clinical nutrition*, vol. *73*(2), pp. 374s-379s, 2001.

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

[14] Malathi, V.B. and Selvakumar, D., 2016. Bacteriocin Production by Lactococcus Lactis MTCC 440.

[15] N. Madi and R. Boushaba, "Identification of Potential Biopreservative Lactic Acid Bacteria Strains Isolated from Algerian Cow's Milk and Demonstration of Antagonism Against S. aureus in Cheese," *Food Science and Technology Research*, vol. *23*(5), pp. 679-688, 2017.

[16] Joint FAO/WHO Expert Committee on Food Additives. Meeting, Joint FAO/WHO Expert Committee on Food Additives and World Health Organization, 2002. *Evaluation of certain mycotoxins in food: fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives* (Vol. 56). World Health Organization.

[17] S. J. Salminen, M. Gueimonde and E. Isolauri, "Probiotics that modify disease risk," *The Journal of nutrition*, vol. *135*(5), pp. 1294-1298, 2005.

[18] A. Kumar, V. Tomer, A. Kaur V. K. Joshi, "Synbiotics: A culinary Art to Creative Health Foods," *International Journal of Food and Fermentation Technology*, vol. *5*(1), p. 1, 2015.

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

[20] P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis and J. I. Gordon, "An obesity-associated gut microbiome with increased capacity for energy harvest," *Nature*, vol. *444*(7122), p. 1027, 2006.

[21] C. Forestier, C. De Champs, C. Vatoux and B. Joly, "Probiotic activities of Lactobacillus caseirhamnosus: in vitro adherence to intestinal cells and antimicrobial properties," *Research in Microbiology*, vol. *152*(2), pp. 167-173, 2001.

[22] R. Al-Tawaha, C. Meng, "Potential benefits of Lactobacillus plantarum as probiotic and its advantages in human health and industrial applications: A review," *Advances in Environmental Biology*, *12*(1), pp.16-27, 2018.

[23] R.Nagpal, A. Kumar, M. Kumar, P.V. Behare, S. Jain, and H. Yadav, "Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS microbiology letters*, *334*(1), pp.1-15, 2012.

[24] M. M. Toma and J. Pokrotnieks, "Probiotics as functional food: microbiological and medical aspects," *Acta Universitatis Latviensis*, vol. *710*, pp. 117-129, 2006.

[25] Y. Ohashi and K. Ushida, "Health‐beneficial effects of probiotics: Its mode of action," *Animal Science Journal*, vol. *80*(4), pp. 361-371, 2009.

[26] A. Ljungh and T. Wadstrom, "Lactic acid bacteria as probiotics," *Current issues in intestinal microbiology*, vol. *7*(2), pp. 73-90, 2006.

[27] M. Kleerebezem, O. P. Kuipers E. J. Smid, "Lactic acid bacteria—a continuing journey in science and application," *FEMS microbiology reviews*, vol. *41*(Supp_1), S1-S2, 2017.

[28] S. E. Evivie, G. C. Huo, J. O. Igene and X. Bian, "Some current applications, limitations and future perspectives of lactic acid bacteria as probiotics," *Food & nutrition research*, vol. *61*(1), 1318034, 2017.

[29] R. M. Bennour, T. C. Ells, F. J. Pagotto, J. M. Farber, A. Kerouanton, T. Meheut, P. Colin, H. Joosten, A. Leclerc and N. G. Bess, "Genotypic and phenotypic characterisation of a collection of Cronobacter (Enterobactersakazakii) isolates," *International Journal of Food microbiology,* vol. *139*(1-2), pp.116-125, 2010.

[30] O. Holý and S. Forsythe, "Cronobacter spp. as emerging causes of healthcare-associated infection," in *Journal of Hospital Infection*, vol. *86*(3), pp. 169-177, 2014.

[31] N. Thakur, S. Bhalla and T. C. Bhalla, "Microbiological and biochemical characterization of experimentally produced Sura-a traditional fermented millet

based alcoholic beverage of Kullu District of Himachal Pradesh, India," *International Journal of Food and Fermentation Technology,* vol. *5*(2), p. 129, 2015.

[32] J. H. Kim, D. E. Block, D. A. Mills, "Simultaneous consumption of pentose and hexose sugars: an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass," *Appl. Microbiol. Biotechnol.*, vol. *88*, pp. 1077–1085, 2010.

[33] X. Bao, X. Jia, L. Chen, B. M. Peters, C. W. Lin, D. Chen and M. E. Shirtliff, "Effect of polymyxin resistance (pmr) on biofilm formation of Cronobactersakazakii," *Microbial pathogenesis*, vol. *106*, pp. 16-19, 2017.

[34] N. Singh, A. Patil, A. Prabhune, G. Goel, "Inhibition of quorum-sensing-mediated biofilm formation in Cronobacter sakazakii strains," *Microbiology*, *162*(9), pp.1708-1714, 2016.

[35] N. Singh, A. Patil, A.A. Prabhune, M. Raghav, and G. Goel, "Diverse profiles of N-acyl-homoserine lactones in biofilm forming strains of Cronobacter sakazakii," *Virulence*, *8*(3), pp.275-281, 2017.

[36] N. Singh, G. Goel and M. Raghav, "Prevalence and characterization of Cronobacter spp. from various foods, medicinal plants, and environmental samples," *Current microbiology*, *71*(1), pp.31-38, 2015.

[37] T. Stiernagle, "Maintenance of C. elegans, WormBook, ed.," *The C. elegans Research Community, WormBook,* February 11, 2006.

[38] C.I. Bargmann, "Chemosensation in C. elegans," 2006.

[39] C. Darby, "Interactions with microbial pathogens," 2005.

[40] R. Zhang and A. Hou, "Host-microbe interactions in Caenorhabditis elegans," *ISRN microbiology*, 2013.

References (Chapter 2)

[1] M. R. Swain, M. Anandharaj, R. C. Ray, R. Parveen Rani, “Fermented fruits and vegetables of Asia: a potential source of probiotics,” *Biotechnology research international,* 2014.

[2] A. Kumari, K. Angmo and T. C. Bhalla, “Probiotic attributes of indigenous Lactobacillus spp. isolated from traditional fermented foods and beverages of north-western Himalayas using in vitro screening and principal component analysis,” *Journal of food science and technology*, *53*(5), pp. 2463-2475, 2016.

[3] T. C. Bhalla, “Traditional foods and beverages of Himachal Pradesh,” *Indian Journal of Traditional Knowledge,* vol. *6*, pp. 17-24, 2007.

[4] T.C. Bhalla, “Characterization of bhatooru, a traditional fermented food of Himachal Pradesh: microbiological and biochemical aspects,” *3 Biotech*, pp. 1-8, 2013.

[5] G. Reid and F. A. O. Joint, “Regulatory and clinical aspects of dairy probiotics,” Cordoba, Argentina: FAO/WHO, 2001.

[6] R. R. Watson and V. R. Preedy, “Probiotics, prebiotics, and synbiotics: bioactive foods in health promotion,” *Academic Press*, 2015.

[7] Z. N. Yuksekdag and B. Aslim, “Assessment of potential probiotic-and starter properties of Pediococcus spp. isolated from Turkish-type fermented sausages (sucuk),” *Journal of Microbiology and Biotechnology*, vol. *20*, pp. 161-168, 2010.

[8] C. R. Soccol, L. P. D. S. Vandenberghe, M. R. Spier, A. B. P. Medeiros, C. T. Yamaguishi, J. D. D. Lindner, A. Pandey, V. Thomaz-Soccol, “The potential of probiotics: a review,” *Food Technology and Biotechnology*, vol. *48,* pp. 413-434, 2010.

[9] O. Lowrey, C. J. Rollins, “Antibiotic-associated diarrhea and probiotics,” *Pharmacy Today,* vol. *18*(7), p. 35, 2012.

[10] N. Sharma, A. Gupta, S. Handa, “An exploration of rich microbial diversity of rare traditional functional foods of Trans Himalayan state of India with proven additional probiotic effect,” International Journal of Current Microbiology and Applied Science, vol. *3,* pp.999-1014, 2014.

[11] K. Angmo, A. Kumari, T. C. Bhalla, “Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh,” *LWT-Food Science and Technology*, vol. *66*, pp. 428-435, 2016.

[12] L. Fontana, M. Bermudez-Brito, J. Plaza-Diaz, S. Munoz-Quezada, A. and Gil, “Sources, isolation, characterisation and evaluation of probiotics,” *British journal of nutrition*, *109*(S2), pp.S35-S50, 2013.

[13] V. Tremaroli, F. Bäckhed, “Functional interactions between the gut microbiota and host metabolism,” *Nature*, *489*(7415), p.242, 2012.

[14] S. Pieniz, R. Andreazza, T. Anghinoni, F. Camargo, A. Brandelli, “Probiotic potential, antimicrobial and antioxidant activities of Enterococcus durans strain LAB18s," *Food Control*, *37*, pp.251-256, 2014.

[15] D. de Oliveira Scoaris, J. Colacite, C.V. Nakamura, T. Ueda-Nakamura, B.A. de Abreu Filho and B.P. Dias Filho,” Virulence and antibiotic susceptibility of Aeromonas spp. isolated from drinking water. *Antonie van Leeuwenhoek*, *93*(1-2), pp.111-122, 2008.

[16] S. Possemiers, K. Verthé, S. Uyttendaele, W. Verstraete, “PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem,” *FEMS Microbiology Ecology*, vol. *49*, pp. 495-507, 2004.

References (Chapter 3)

[1] G. Reid and F. A. O. Joint, "Regulatory and clinical aspects of dairy probiotics," Cordoba, Argentina: FAO/WHO, 2001.

[2] R. R. Watson and V. R. Preedy, "Probiotics, Prebiotics, and Synbiotics: Bioactive Foods in Health Promotion," *Academic Press*, 2015.

[3] Z. N. Yuksekdag and B. Aslim, "Assessment of potential probiotic-and starter properties of *Pediococcus* spp. isolated from Turkish-type fermented sausages (sucuk)," *J. Microbiol. Biotechnol,* vol. *20*, pp. 161-168, 2010.

[4] C. R. Soccol, L. P. D. S. Vandenberghe, M. R. Spier, A. B. P. Medeiros, C. T. Yamaguishi, J. D. D. Lindner, A. Pandey, V. Thomaz-Soccol, "The potential of probiotics: a review," *Food Technology and Biotechnology,* vol. *48,* pp. 413-434, 2010.

[5] O. Lowrey, C. J. Rollins, "Antibiotic-associated diarrhea and probiotics," *Pharmacy Today*, vol. *18*(7), p. 35, 2012.

[6] K. Jampaphaeng, L. Cocolin, S. Maneerat, "Selection and evaluation of functional characteristics of autochthonous lactic acid bacteria isolated from traditional fermented stinky bean (Satawdong)," *Annals of Microbiology,* vol. *67*(1), pp. 25–36, 2017.

[7] M. L. Marco, D. Heeney, S. Binda, C. J. Cifelli, P. D. Cotter, B. Foligne, E. J. Smid, "Health benefits of fermented foods: microbiota and beyond," Current Opinion in Biotechnology, vol. *44*, pp. 94–102, 2017.

[8] F. Manini, M. C. Casiraghi, K. Poutanen, M. Brasca, D. Erba and C. Plumed-Ferrer, "Characterization of lactic acid bacteria isolated from wheat bran sourdough," *LWT-food Science and Technology*, vol. *66*, pp. 275-283, 2016.

[9] T. C. Bhalla, "Characterization of bhatooru, a traditional fermented food of Himachal Pradesh: microbiological and biochemical aspects," *3 Biotech*, vol. *3*(3), pp. 247-254, 2013.

[10] N. Sharma, S. Handa, A. Gupta, "Comprehensive study of different traditional fermented foods/beverages of himachal pradesh to evaluate their nutrition impact on

health and rich biodiversity of fermenting microorganisms," *International Journal of Research in Applied Natural and Social Sciences*, vol. *1*, pp. 19-28, 2013.

[11] M. Ray, K. Ghosh, S. Singh and K. C. Mondal, "Folk to functional: an explorative overview of rice-based fermented foods and beverages in India," *Journal of Ethnic Foods*, vol. *3*(1), pp. 5-18, 2016.

[12] P. L. Conway, S. L. Gorbach and B. R. Goldin, "Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells," *Journal of dairy science*, vol. *70*(1), pp. 1-12, 1987.

[13] B. Del Re, B. Sgorbati, M. Miglioli and D. Palenzona, "Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum," *Letters in applied microbiology*, vol. *31*(6), pp. 438-442, 2000.

[14] M. Rosenberg, D. Gutnick and E. Rosenberg, "Adherence of bacteria to hydrocarbons: a simple method for measuring cell‐surface hydrophobicity," *FEMS microbiology letters*, vol. *9*(1), pp. 29-33, 1980.

[15] D. Abedi, S. Feizizadeh, V. Akbari and A. Jafarian-Dehkordi, "In vitro anti-bacterial and anti-adherence effects of Lactobacillus delbrueckii subsp bulgaricus on Escherichia coli," *Research in pharmaceutical sciences*, vol. *8*(4), p. 260, 2013.

[16] A. Balakrishna, "In vitro evaluation of adhesion and aggregation abilities of four potential probiotic strains isolated from guppy (Poecilia reticulata)," *Brazilian Archives of Biology and Technology*, vol. *56*(5), pp. 793-800, 2013.

[17] A. E. M. M. Afify, R. M. Romeilah, S. I. Sultan and m. M. Hussein, "Antioxidant activity and biological evaluations of probiotic bacteria strains," *International Journal of Academic Research*, vol. *4*(6), 2012.

[18] J. K. Kaushik, A. Kumar, R. K. Duary, A. K. Mohanty, S. Grover and V. K. Batish, "Functional and probiotic attributes of an indigenous isolate of Lactobacillus plantarum," *PloS one*, vol. *4*(12), e8099, 2009.

[19] K. Angmo, A. Kumari T. C. Bhalla, "Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh," *LWT-food Science and Technology*, vol. *66*, pp. 428-435, 2016.

[20] K. W. Lee, J. M. Shim, S. K. Park, H. J. Heo, H. J. Kim, K. S. Ham and J. H. Kim, "Isolation of lactic acid bacteria with probiotic potentials from kimchi, traditional

Korean fermented vegetable," *LWT-Food Science and Technology*, vol. *71*, pp. 130-137, 2016.

[21] S. H. Son, H. L. Jeon, S. J. Yang, M. H. Sim, Y. J. Kim, N. K. Lee and H. D. Paik, "Probiotic lactic acid bacteria isolated from traditional Korean fermented foods based on β-glucosidase activity," *Food Science and Biotechnology*, vol. *27*(1), pp. 123-129, 2018.

[22] M. C. De Vries, E. E. Vaughan, M. Kleerebezem and W. M. de Vos, "Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract," *International Dairy Journal*, vol. *16*(9), pp. 1018-1028, 2006.

[23] N. Rokana, R. H. Mallappa, V. K. Batish and S. Grover, "Interaction between putative probiotic Lactobacillus strains of Indian gut origin and Salmonella: Impact on intestinal barrier function," *LWT-Food Science and Technology*, vol. *84*, pp. 851-860, 2017.

[24] L. Fontana, M. Bermudez-Brito, J. Plaza-Diaz, S. Munoz-Quezada and A. Gil, "Sources, isolation, characterisation and evaluation of probiotics," *British journal of nutrition*, vol. *109*(S2), pp. S35-S50, 2013.

[25] B. R. Goldin, S. L. Gorbach, M. Saxelin, S. Barakat, L. Gualtieri and S. Salminen, "Survival ofLactobacillus species (strain GG) in human gastrointestinal tract," *Digestive diseases and sciences*, vol. *37*(1), pp. 121-128, 1992.

[26] D. Bakari, N. L. Tatsadjieu, A. Mbawala C. M. Mbofung, "Assessment of physiological properties of some lactic acid bacteria isolated from the intestine of chickens use as probiotics and antimicrobial agents against enteropathogenic bacteria," *Innovative Romanian Food Biotechnology*, vol. *8*, 33, 2011.

[27] N. Rokana, R. H. Mallappa, V. K. Batish and S. Grover, "Interaction between putative probiotic Lactobacillus strains of Indian gut origin and Salmonella: Impact on intestinal barrier function," *LWT-Food Science and Technology*, vol. *84*, pp. 851-860, 2017.

[28] A. C. Ouwehand, P. V. Kirjavainen, C. Shortt and S. Salminen, "Probiotics: mechanisms and established effects," *International dairy journal*, vol. *9*(1), pp. 43-52, 1999.

[29] C. G. Vinderola, M. Medici and G. Perdigon, "Relationship between interaction sites in the gut, hydrophobicity, mucosal immunomodulating capacities and cell wall protein profiles in indigenous and exogenous bacteria," *Journal of Applied Microbiology*, vol. *96*(2), pp. 230-243, 2004.

[30] M. S. R. Rajoka, H. M. Mehwish, M. Siddiq, Z. Haobin, J. Zhu, L. Yan and J. Shi, "Identification, characterization, and probiotic potential of Lactobacillus rhamnosus isolated from human milk," *LWT-Food Science and Technology*, vol. *84*, pp. 271-280, 2017.

[31] K. Nishiyama, M. Sugiyama and T. Mukai, "Adhesion properties of lactic acid bacteria on intestinal mucin," *Microorganisms*, vol. *4*(3), p. 34, 2016.

[32] E. Korcz, Z. Kerényi and L. Varga, "Dietary fibers, prebiotics, and exopolysaccharides produced by lactic acid bacteria: potential health benefits with special regard to cholesterol-lowering effects," *Food & function*, vol. *9*(6), pp. 3057-3068, 2018.

[33] M. Rosenberg, "Microbial adhesion to hydrocarbons: twenty-five years of doing MATH," *FEMS microbiology letters*, vol. *262*(2), pp. 129-134, 2006.

[34] D. Bujnakova and V. Kmet, "Aggregation of animal lactobacilli with O157 enterohemorrhagic Escherichia coli," *Journal of Veterinary Medicine*, *Series B*, vol. *49*(3), pp. 152-154, 2002.

[35] F. Dal Bello, J. Walter, C. Hertel and W. P. Hammes, "In vitro study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis," *Systematic and Applied Microbiology*, vol. *24*(2), pp. 232-237,2 001.

[36] Y. Wang, Y. Wu, Y. Wang, H. Xu, X. Mei, D. Yu and W. Li, "Antioxidant properties of probiotic bacteria," *Nutrients*, vol. *9*(5), p. 521, 2017.

[37] J. Yang, J. Guo and J. Yuan, "In vitro antioxidant properties of rutin," *LWT-Food Science and Technology*, vol. *41*(6), pp. 1060-1066, 2008.

[38] J. Zhang, D. Wang, J. Zhang and Y. H. Wang, "Extraction and antioxidant activity of the soluble protein from Lactobacillus rhamnosus broth," *Food Sci Technol*, vol. *37*, pp. 235-239, 2012.

[39] L. Zhang, C. Liu, D. Li, Y. Zhao, X. Zhang, X. Zeng and S. Li, “Antioxidant activity of an exopolysaccharide isolated from Lactobacillus plantarum C88,” *International Journal of Biological Macromolecules*, vol. *54*, pp. 270-275, 2013.

[40] L. Noriega, I. Cuevas, A. Margolles and G. Clara, “Deconjugation and bile salts hydrolase activity by Bifidobacterium strains with acquired resistance to bile,” *International dairy journal*, vol. *16*(8), pp. 850-855, 2006.

[41] M. Kumar, R. Nagpal, R. Kumar, R. Hemalatha, V. Verma, A. Kumar and H. Yadav, “Cholesterol-lowering probiotics as potential biotherapeutics for metabolic diseases,” *Experimental diabetes research*, 2012.

[42] S. Galle and E. K. Arendt, “Exopolysaccharides from sourdough lactic acid bacteria,” *Critical reviews in food science and nutrition*, vol. *54*(7), pp. 891-901, 2014.

References (Chapter 4)

[1] P. Sornplang and S. Piyadeatsoontorn, “Probiotic isolates from unconventional sources: a review,” *Journal of Animal Science and Technology*, vol. *58*(1), p. 26, 2016.

[2] A. C. Ouwehand and S. Salminen, “In vitro adhesion assays for probiotics and their *in vivo* relevance: a review,” *Microbial Ecology in Health and Disease*, vol. *15*(4), pp. 175-184, 2003.

[3] J. C. Byrd and R. S. Bresalier, “Mucins and mucin binding proteins in colorectal cancer,” *Cancer and Metastasis Reviews*, vol. *23*(1-2), pp.77-99, 2004.

[4] M. L. Van Tassell and M. J. Miller, “Lactobacillus adhesion to mucus,” *Nutrients*, vol. *3*(5), pp. 613-636, 2011.

[5] G. Saxami, A. Karapetsas, E. Lamprianidou, I. Kotsianidis, A. Chlichlia, C. Tassou, V. Zoumpourlis and A. Galanis, “Two potential probiotic lactobacillus strains isolated from olive microbiota exhibit adhesion and anti-proliferative effects in cancer cell lines,” *Journal of Functional Foods*, vol. *24*, pp. 461-471, 2016.

[6] R. K. Duary, Y. S. Rajput, V. K. Batish and S. Grover, “Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells,” *The Indian journal of medical research*, vol. *134*(5), p. 664, 2011.

[7] V. D. Valeriano, M. M. Parungao‐Balolong and D. K. Kang, “In vitro evaluation of the mucin‐adhesion ability and probiotic potential of L actobacillus mucosae LM 1,” *Journal of applied microbiology*, vol. *117*(2), 485-497, 2014.

[8] P. Carasi, M. Díaz, S. M. Racedo, G. De Antoni, M. C. Urdaci and M. D. L. A. Serradell, “Safety characterization and antimicrobial properties of kefir-isolated Lactobacillus kefiri,” BioMed research international, 2014.

[9] T. Ikeda, C. Yasui, K. Hoshino, K. Arikawa and Y. Nishikawa, “Influence of lactic acid bacteria on longevity of Caenorhabditis elegans and host defense against Salmonella enterica serovar enteritidis,” *Applied and environmental microbiology*, vol. *73*(20), pp. 6404-6409, 2007.

[10] M. R. Park, H. S. Yun, S. J. Son, S. Oh Y. Kim, “Development of a direct in vivo screening model to identify potential probiotic bacteria using Caenorhabditis elegans,” *Journal of dairy science*, vol. *97*(11), pp. 6828-6834, 2014.

[11] M. C. Leung, P. L. Williams, A. Benedetto, C. Au, K. L. Helmcke, M. Aschner and J. N. Meyer, “Caenorhabditis elegans: an emerging model in biomedical and environmental toxicology,” Toxicological sciences, vol.*106*(1), pp. 5-28, 2008.

[12] J. D. McGhee, M. C. Sleumer, M. Bilenky, K. Wong, S. J. McKay, B. Goszczynski and D. L. Baillie, “The ELT-2 GATA-factor and the global regulation of transcription in the C. elegans intestine,” *Developmental biology*, vol. *302*(2), pp. 627-645, 2007.

[13] G. Grompone, P. Martorell, S. Llopis, N. González, S. Genovés, A. P. Mulet and B. Foligné, “Anti-inflammatory Lactobacillus rhamnosus CNCM I-3690 strain protects against oxidative stress and increases lifespan in Caenorhabditis elegans,” *PloS one*, vol. *7*(12), e52493, 2012.

[14] K. Sharma, R. Mahajan, S. Attri and G. Goel, “Selection of indigenous Lactobacillus paracasei CD 4 and Lactobacillus gastricus BTM 7 as probiotic: assessment of traits combined with principal component analysis,” *Journal of applied microbiology*, vol. *122*(5), pp. 1310-1320, 2017.

[15] S. Brenner, “The genetics of Caenorhabditis elegans,” *Genetics*, vol. *77*(1), pp. 71-94, 1974.

[16] E. L. Kaplan and P. Meier, “Nonparametric estimation from incomplete observations,” *Journal of the American statistical association*, vol. *53*(282), pp. 457-481, 1958.

[17] E. A. E. Abada, H. Sung, M. Dwivedi, B. J. Park, S. K. Lee and J. Ahnn, “C. elegans behavior of preference choice on bacterial food,” *Molecules and cells*, vol. *28*(3), pp. 209-213, 2009.

[18] S. Durai, S. K. Pandian and K. Balamurugan, “Establishment of a Caenorhabditis elegans infection model for Vibrio alginolyticus,” *Journal of basic microbiology*, vol. *51*(3), pp. 243-252, 2011.

[19] N. K. Lee, K. J. Han, S. H. Son, S. J. Eom, S. K. Lee and H. D. Paik, "Multifunctional effect of probiotic Lactococcus lactis KC24 isolated from kimchi," *LWT-Food Science and Technology*, vol. *64*(2), pp. 1036-1041, 2015.

[20] D. Ghosh and P. Chattopadhyay, "Application of principal component analysis (PCA) as a sensory assessment tool for fermented food products," *Journal of food science and technology*, vol. *49*(3), pp. 328-334, 2012.

[21] E. K. Marsh and R. C. May, "Caenorhabditis elegans, a model organism for investigating immunity, "*Applied and Environmental Microbiology*, vol. *78*(7), pp. 2075-2081, 2012.

[22] I. I. Metchnikoff, "The prolongation of life: optimistic studies," *Springer Publishing Company*, 2004.

[23] T. Shintani, H. Sakoguchi, A. Yoshihara, K. Izumori and M. Sato, "D-Allulose, a stereoisomer of D-fructose, extends Caenorhabditis elegans lifespan through a dietary restriction mechanism: A new candidate dietary restriction mimetic," *Biochemical and biophysical research communications*, vol. *493*(4), 1528-1533, 2017.

[24] L. Zhao, Y. Zhao, R. Liu, X. Zheng, M. Zhang, H. Guo and F. Ren, "The transcription factor DAF-16 is essential for increased longevity in C. elegans exposed to Bifidobacterium longum BB68," *Scientific reports*, vol. *7*(1), 7408, 2017.

[25] Y. Kim and E. Mylonakis, "Caenorhabditis elegans immune conditioning with the probiotic bacterium Lactobacillus acidophilus NCFM enhances gram-positive immune responses," *Infection and immunity*, IAI-06350, 2012.

[26] J. Lee, H. S. Yun, K. W. Cho, S. Oh, S. H. Kim, T. Chun and K. Y. Whang, "Evaluation of probiotic characteristics of newly isolated Lactobacillus spp.: immune modulation and longevity," *International journal of food microbiology*, vol. *148*(2), pp. 80-86, 2011.

[27] H. Nakagawa, T. Shiozaki, E. Kobatake, T. Hosoya, T. Moriya, F. Sakai and T. Miyazaki, "Effects and mechanisms of prolongevity induced by Lactobacillus gasseri SBT2055 in Caenorhabditis elegans," *Aging cell*, vol. *15*(2), pp. 227-236, 2016.

[28] R. Azat, Y. Liu, W. Li, A. Kayir, D. B. Lin, W. W. Zhou X. D. Zheng, “Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese,” *Journal of Zhejiang University-SCIENCE B*, vol. *17*(8), pp. 597-609, 2016.

[29] T. Komura, T. Ikeda, C. Yasui, S. Saeki and Y. Nishikawa, “Mechanism underlying prolongevity induced by bifidobacteria in Caenorhabditis elegans,” *Biogerontology*, vol. *14*(1), pp. 73-87, 2013.

[30] H. K. Lee, S. H. Choi, C. R. Lee, S. H. Lee, M. R. Park, Y. Kim and G. B. Kim, “Screening and characterization of lactic acid bacteria strains with anti-inflammatory activities through in vitro and Caenorhabditis elegans model testing,” *Korean journal for food science of animal resources*, vol. *35*(1), p. 91, 2015.

[31] J. Im Choi, K. H. Yoon, S. S. Kalichamy, S. S. Yoon and J. I. Lee, “A natural odor attraction between lactic acid bacteria and the nematode Caenorhabditis elegans,” *The ISME journal*, vol. *10*(3), p. 558, 2016.

[32] C. I. Bargmann, E. Hartwieg and H. R. Horvitz, “Odorant-selective genes and neurons mediate olfaction in C. elegans,” *Cell*, vol. *74*(3), pp. 515-527, 1993.

[33] N. F. Trojanowski, D. M. Raizen and C. Fang-Yen, “Pharyngeal pumping in Caenorhabditis elegans depends on tonic and phasic signaling from the nervous system,” *Scientific reports*, vol. *6*, 22940, 2016.

[34] B. M. Song and L. Avery, “The pharynx of the nematode C. elegans: a model system for the study of motor control,” in *Worm, Taylor & Francis*, Vol. *2*(1), pp. 1920-1931, 2013.

[35] C. Mörck and M. Pilon, “C. elegans feeding defective mutants have shorter body lengths and increased autophagy,” *BMC developmental biology*, vol. *6*(1), p. 39, 2006.

[36] M. De Vrese and J. Schrezenmeir, “Probiotics, prebiotics, and synbiotics,” *Food biotechnology*, pp. 1-66, Springer, Berlin, Heidelberg, 2008.

References (Chapter 5)

[1] N. Singh, G. Goel and M. Raghav, "Prevalence and characterization of Cronobacter spp. from various foods, medicinal plants, and environmental samples," *Current microbiology*, vol. *71*(1), pp. 31-38, 2015.

[2] J. Van Gestel, H. Vlamakis and R. Kolter, "From cell differentiation to cell collectives: Bacillus subtilis uses division of labor to migrate," *PLoS biology*, vol. *13*(4), p.e1002141, 2015.

[3] M. Hayes, R. P. Ross, G. F. Fitzgerald, C. Hill and C. Stanton, "Casein-derived antimicrobial peptides generated by Lactobacillus acidophilus DPC6026," *Applied and environmental microbiology*, vol. *72*(3), pp. 2260-2264, 2006.

[4] T. Aymerich, P. A. Picouet and J. M. Monfort, "Decontamination technologies for meat products," *Meat Science*, vol. *78*(1-2), pp. 114-129, 2008.

[5] T. Ikeda, C. Yasui, K. Hoshino, K. Arikawa and Y. Nishikawa, "Influence of lactic acid bacteria on longevity of Caenorhabditis elegans and host defense against Salmonella enterica serovar enteritidis," *Applied and environmental microbiology*, vol. *73*(20), pp. 6404-6409, 2007.

[6] J. R. Tagg, A. S. Dajani and L. W. Wannamaker, "Bacteriocins of gram-positive bacteria," *Bacteriological reviews*, vol. *40*(3), p. 722, 1976.

[7] S. S. Awaisheh and S. A. Ibrahim, "Screening of antibacterial activity of lactic acid bacteria against different pathogens found in vacuum-packaged meat products," *Foodborne pathogens and disease*, vol. *6*(9), pp. 1125-1132, 2009.

[8] T. I. Moy, A. R. Ball, Z. Anklesaria, G. Casadei, K. Lewis and F. M. Ausubel, "Identification of novel antimicrobials using a live-animal infection model," *Proceedings of the National Academy of Sciences*, vol. *103*(27), pp. 10414-10419, 2006.

[9] N. Singh, G. Goel and M. Raghav, "Insights into virulence factors determining the pathogenicity of Cronobacter sakazakii," *Virulence*, vol. *6*(5), pp. 433-440, 2015.

[10] S. Durai, S. K. Pandian and K. Balamurugan, "Establishment of a Caenorhabditis elegans infection model for Vibrio alginolyticus," *Journal of basic microbiology*, vol. *51*(3), pp. 243-252, 2011.

[11] K. Sharma, S. Attri and G. Goel, “Selection and Evaluation of Probiotic and Functional Characteristics of Autochthonous Lactic Acid Bacteria Isolated from Fermented Wheat Flour Dough Babroo,” *Probiotics and antimicrobial proteins*, pp.1-11, 2018.

[12] K. Sharma, R. Mahajan, S. Attri and G. Goel, “Selection of indigenous Lactobacillus paracasei CD 4 and Lactobacillus gastricus BTM 7 as probiotic: assessment of traits combined with principal component analysis,” *Journal of applied microbiology*, vol. *122*(5), pp. 1310-1320, 2017.

[13] B. A. Adeniyi, A. Adetoye and F. A. Ayeni, “Antibacterial activities of lactic acid bacteria isolated from cow faeces against potential enteric pathogens,” *African health sciences*, vol. *15*(3), pp. 888-895, 2015.

[14] L. Baldassarri, R. Creti, S. Recchia, M. Imperi, B. Facinelli, E. Giovanetti, M. Pataracchia, G. Alfarone and G. Orefici, “Therapeutic failures of antibiotics used to treat macrolide-susceptible Streptococcus pyogenes infections may be due to biofilm formation,” *Journal of Clinical Microbiology*, vol. *44*(8), pp. 2721-2727, 2006.

[15] R. Thenmozhi, P. Nithyanand, J. Rathna and S. Karutha Pandian, “Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of Streptococcus pyogenes,” *FEMS Immunology & Medical Microbiology*, vol. *57*(3), pp. 284-294, 2009.

[16] K. Musthafa, A. Ravi, A. Annapoorani, I. V. Packiavathy and S. Pandian, “Evaluation of anti-quorum-sensing activity of edible plants and fruits through inhibition of the N-acyl-homoserine lactone system in Chromobacterium violaceum and Pseudomonas aeruginosa,” Chemotherapy, vol. *56*, pp. 333–339, 2010.

[17] N. Singh, A. Patil, A. Prabhune and G. Goel, “Inhibition of quorum-sensing-mediated biofilm formation in Cronobacter sakazakii strains,” *Microbiology*, vol. *162*(9), pp. 1708-1714, 2016.

[18] B. S. Sivamaruthi, A. Ganguli, M. Kumar, S. Bhaviya, S. K. Pandian and K. Balamurugan, “Caenorhabditis elegans as a model for studying Cronobacter sakazakii ATCC BAA‐894 pathogenesis,” *Journal of basic microbiology*, vol. *51*(5), pp. 540-549, 2011.

[19] K.. Sharma, M. Pooranachithra, K. Balamurugan and G. Goel, "Multivariate Analysis of Increase in Life Span of Caenorhabditis elegans Through Intestinal Colonization by Indigenous Probiotic Strains," *Probiotics and antimicrobial proteins*, pp. 1-9, 2018.

[20] M. I. Prasanth, G. S. Santoshram, J. P. Bhaskar and K. Balamurugan, "Ultraviolet-A triggers photoaging in model nematode Caenorhabditis elegans in a DAF-16 dependent pathway," *Age*, vol. *38*(1), p. 27, 2016.

[21] J. Woo and J. Ahn, "Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens," *Letters in applied microbiology*, vol. *56*(4), pp. 307-313, 2013.

[22] E. Yang, L. Fan, Y. Jiang, C. Doucette and S. Fillmore, "Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts," *Amb Express*, vol. *2*(1), p. 48, 2012.

[23] R. Denkova, Z. Denkova, V. Yanakieva and D. Blazheva, "Antimicrobial activity of probiotic lactobacilli, bifidobacteria and propionic acid bacteria, isolated from different sources," *Microbial pathogens and strategies for combating them: science, technology and education*, pp. 857-864, 2013.

[24] H. L. Alakomi, E. Skyttä, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala and I. M. Helander, "Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane," *Applied and environmental microbiology*, vol. *66*(5), pp. 2001-2005, 2000.

[25] F. Bendali, N. Madi and D. Sadoun, "Beneficial effects of a strain of Lactobacillus paracasei subsp. paracasei in Staphylococcus aureus-induced intestinal and colonic injury," *International Journal of Infectious Diseases*, vol. *15*(11), pp. e787-e794, 2011.

[26] J. L. Balcázar, D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, J. L. Muzquiz and O. Girones, "Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish," *Aquaculture*, vol. *278*(1-4), pp. 188-191, 2008.

[27] Y. Kim and E. Mylonakis, "Caenorhabditis elegans immune conditioning with the probiotic bacterium Lactobacillus acidophilus strain NCFM enhances gram-positive immune responses," *Infection and immunity*, vol. *80*(7), pp. 2500-2508, 2012.

[28] S. Joseph, E. Cetinkaya, H. Drahovska, A. Levican, M. J. Figueras and S. J. Forsythe, “Cronobactercondimenti sp. nov., isolated from spiced meat, and Cronobacteruniversalis sp. nov., a species designation for Cronobacter sp. genomospecies 1, recovered from a leg infection, water and food ingredients,” *International journal of systematic and evolutionary microbiology*, vol. *62*(6), pp. 1277-1283, 2012.

[29] G. Kalyantanda, L. Shumyak and L. K. Archibald, “Cronobacter species contamination of powdered infant formula and the implications for neonatal health,” *Frontiers in pediatrics*, vol. *3*, p.56, 2015.

[30] B. S. Sivamaruthi, A. Ganguli, M. Kumar, S. Bhaviya, S. K. Pandian and K. Balamurugan, “Caenorhabditis elegans as a model for studying Cronobacter sakazakii ATCC BAA‐894 pathogenesis,” *Journal of basic microbiology*, vol. *51*(5), pp. 540-549, 2011.