ANTIMICROBIAL ACTIVITY OF CELL FREE SUPERNATANT OF INDIGENEOUS LACTIC ACID BACTERIA AGAINST *Cronobacter sakazakii*

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CERTIFICATE

This is to certify that the work presented in this dissertation entitled "**Antimicrobial activity of cell free supernatant of indigenous lactic acid bacteria against *Cronobacter sakazakii*** " was carried out by Ms. Surabhi Chauhan at Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat (Solan) under my supervision towards the partial fulfillment of their Master of Technology in biotechnology. It is also certified that no part of this dissertation has been submitted elsewhere for award of any degree or diploma.

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ABSTRACT

C.sakazakii is an opportunistic pathogen usually found in powdered infant formula (PIF) and causes severe infant diseases like meningitis and bacteremia. As the pathogen is usually encountered in infant formulae therefore, potential of lactic acid bacteria which is common inhabitant of human gut and used a starter in different fermented food preparation was evaluated for its inhibition. A total of nine different strains of Lactobacillus sp., isolated from different fermented food products of Himachal Pradesh were evaluated against different strains of C.sakazakii (ATCC 12868, E604, and lab strains i.e. N112, N15, N13) . The filter sterilized cell free supernatant (CFS) of the lactic cultures grown in MRS broth was used for determination of minimum inhibitory concentration against different strains of C.sakazakii. The MIC was determined using microtiter plate assay with 150 µl Tryptone Soy Broth and 10µl overnight grown C.sakazakii, followed by addition of 0-50µl of CFS of different lactic cultures. The other Lactobacillus sp. strains resulted in reduction of C.sakazakii by 79-86% at 50µl. Further, the antimicrobial activity was assessed by agar well assay where the strains 94,98 and 90 showed significant zone of inhibition ranging between 15-20mm. Further studies are in progress to elucidate the active moiety in the CFS for its antimicrobial activities. To further look at the potential component for antimicrobial activity ammonium sulphate precipitation was performed,and it was seen that a clear zone of inhibition was observed for 0-40% saturation against E604. Thereby, confirming that bacteriocins exhibited the antimicrobial activity. The presence of protein was confirmed by SDS PAGE which showed clear results of presence of protein at 0-40% saturation.

ABBREVIATIONS

ml.	**mililetres**
L	**litres**
º	**degrees**
µ	**micro**
gm	**gram**
rpm	**revolution per minute**
CFS	**cell free supernatant**
LAB	**lactic acid bacteria**
MIC	**minimum inhibitory concentration**
PIF	**powdered infant formula**
mm	**milimetre**
µg	**microgram**
µl	**microlitre**
mg	**milligrams**

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Nutritional composition is the main feature of food as it determines its consumption among vigilant consumer. The main concern of customer during dealing with food and food produces lies in the nutritional composition of food. Also it has to be kept in mind that the food should be free from any infection causing agents and that they should be destroyed before the food is consumed. As the era of packaged foods, cereals is progressing a new found danger in food items is establishing itself and that danger is from food borne pathogens. One such pathogen in food is *Cronobacter sakazakii* which is a ubiquitous, gram negative, motile, non-spore forming, pervasive in food produces like powdered infant formula, fruits, processed foods, spices, soil, rain water, beverages etc.

Cronobacter spp. are among the most harmful food borne pathogens and include eleven species *Cronobacter muytjensii, Cronobacter condimenti Cronobacter sakazakii, Cronobacter helveticus, Cronobacter zurichenesis Cronobacter malonaticus, Cronobacter dublinensis, Cronobacter turicensis, , Cronobacter universalis, Cronobacter pulveris* and *Cronobacter colletis* Flores et al., (2016) of which *Cronobacter sakazakii* is the major reason for severe diseases like bacteremia, meningitis(bacteria in the meninges),septicemia majorly in neonates (Ye et al., 2016).

Till date, cases of Cronobacter infections in neonates and young children have been reported. The source of *C. sakazakii* is not defined priory as the organism is said to be everywhere soil,rain water, further the studies implicate reconstituted powdered infant formula (PIF) as the main source of transmission (Healy et al., 2010).

This pathogen forms a biofilm which helps it escape from pasteurization stresses during packaging. This biofilm is a technique which is the possible way of infection into food items to cause infection. Biofilms have numerous colonies of microorganisms in them. The biofilm when gets removed from the abiotic

surface individual microorganisms can easily spread. These organisms that now leave the surface and spread are more resistant to any kind of stresses during food packaging (Brown et al., 1999 and Davey et al., 2000).

The biofilm has been found to form due to the presence of EPS (extracellular polymeric substances). *C. sakazakii* has been found to have great resistance to drying at 25ºC - 45ºC than various other species. This resistance to desiccation is brought about by presence of EPS in biofilm (Jung et al., 2012).

Considering the harmful effects the pathogen could pose, it has become necessary to combat its effects. Many compounds, formulations have been checked to calculate the antimicrobial activity also the physical removal using force is quite prevalent.

Chemical disinfectants

(i) Oxidising agents such as hydrogen peroxide, ozone and PAA,

(ii) surface-active compounds

(iii) Also the effect of pH, temperature and quorum sensing is being explored

Physical methods

Ultrasound, electric fields have been explored as potential techniques to evict biofilms (Van et al., 2010).

Other methods

Other methods like use of probiotic cultures, varying atmospheric conditions like concentration of nitrogen, oxygen. Use of copper sulphate is also said to have an inhibiting effect on biofilm. Calcium magnesium ions, lactic cultures from different places, kefir grains originated in the Caucasus mountains, trans-cinnamaldehyde, red muscadine juice, blueberry proanthocyanidins and tea polyphenols have been studied to inhibit the pathogen to a great extent.

Tannic acid in muscadine juice has been studied to show the highest inhibition. Approximately a 4 log reduction within 2 hrs. (Kim et al., 2010).A reduction in the growth on treatment with blueberry

procyanidins was seen and this as a whole reduced within an interval of 7 hrs. from treatment 8.255 log CFU/ml after 1h with 5mg/ml blueberry PAC and BJ after 3h and 6h >8.58 reduction (Joshi et al., 2014).

The tea polyphenols when acidified with HCl had totally inhibited *C.sakazakii* after 7h with an increase in the antimicrobial effect. Cells treated with Tea Polyphenols were seriously damaged and plasma leaked out thus the effect being bactericidal (Li et al., 2016).

The activity of sodium caseinate fermentate was calculated for decreasing the numbers of Cronobacter spp. in reconstituted PIF. At higher final concentrations of fermentate (3.33% w/v), numbers were completely eliminated from 6 logs CFU/ml over a time of 60 min.

Awaisheh et al. (2013) neutralized pH of cell-free supernatant (CFS) of *L. acidophilus* or *L. casei* (isolated from faeces of infants) has antimicrobial activity.

Lactic acid bacteria (LAB) are important starter cultures because of their fermentation skills. LAB has attained the GRAS (generally recognized as safe) status. Mainly the LAB exhibits their effects because of the bacteriocins and pH activity.

The objective of this study was to screen the antimicrobial activity of lactic acid bacteria (LAB) isolated from different sources of fermented foods of Himachal Pradesh and curds and milk of different places against different *C.skazakii* isolates (ATCC 12868, E604, and lab strains i.e. N112, N15, N13). A total of sixteen different strains of Lactobacillus sp., isolated from different fermented food products of Himachal Pradesh.

Thus, this study determines the antimicrobial activity of lactic cultures on *C.sakazakii* and identifies the potential components exhibiting this antimicrobial activity.

This project is being performed to find out the lactic cultures that exhibit this activity .The objective of the study:

Objective: To evaluate the antimicrobial activity of lactic acid bacteria against *C. sakazakii*

CHAPTER 2

REVIEW OF LITERTURE

2. REVIEW OF LITERATURE

2.1 *Cronobacter sakazakii*

Cronobacter sakazakii is an opportunistic motile bacterium which is gram negative in nature. It is a member of enterobacteriaceae which is found to cause infections in neonates and infants. Infections caused by the Cronobacter are disastrous including meningitis, pneumonia, septicemia, osteomyelitis, wound infections. The organism is omnipresent in nature, and found in mainly in powdered infant formula. Other than that it has been found in dried fruits, meat, fish etc. (Fakruddin et al., 2014).

Figure 1: Powdered infant formula

2.1.1 Classification

C.sakazakii was referred as Enterobacter cloacae because it was found to lie in the twilight zone between *Enterobacter* and *citrobacter* lying closer to *Enterobacter* it was given the name later a separate genus

Cronobacter was created to add the pathogen to it. The genus includes eleven species .*C. sakazakii, C.malonaticus, C.helveticus, C.zurichenesi, C.dublinensis, C.turicensis, C.muytjensii, C. condimenti, C.universalis, C.pulveris and C.colletis* Singh et al., (2015) of which *C. sakazakii* is the most harmful.

2.1.2 Reservoirs

Cronobacter species has been isolated from milk, cheese, food items, tea, herbs, infant formula, household, livestock facilities, food factories, PIF production facilities, u skin wounds plants. Also all the additives added to powdered infant formula, beverages without heat treatment may contain traces of the bacteria

A study of collection of at least 219 samples from various locations like environment, spices and food etc. and after the growth of colonies on enrichment media the colonies showed presence of the bacteria. They were selected and isolated on TSA and further plating on chromogenic media E. sakazakii agar. The isolates in this study were compared by genome specific PCR and antibiotic susceptibility test.

Raw milk, gram flour, opened packet of PIF, spices environment showed the presence of the pathogen. 9% presence was seen in raw milk, 34% in herbs and spices, 23% was found in the environment. All the isolated culture was resistant to b-lactam derivatives (68 %), macrolides (88.6 %), and aminoglycosides (79.9 %) but susceptible to phenicoles (31.6 %) and tetracycline's (15 %) derivatives.

It is seen in general sense that a single method off distinguishing the pathogen is not sufficient (Singh et al., 2015).

2.1.3 Stress tolerating strains from food

The following study was performed on food samples containing virulent strains from Bangladesh.

The *C. sakazakii* was sensitive to vancomycin, ampicillin, nitrofurantonin, penincillin G, Imipenem. Serum tolerance test was done to check for virulence properties in which 6 out of 3 isolates were capable

of biofilm formation. Besides this serological cross reactivity of the species with salmonella, shigella boydii, and Vibrio cholera was seen. Virulence genes OmpA (outer membrane protein A) and zpx was seen.

Stress tolerance

Four (66.67%) isolated Cronobacter sakazakii were able to grow at 10% NaCl concentration. MP04.1 and HR11.3 both were able to grow at 7%.

Bile salt tolerance

5% bile salt concentration was easily tolerated by the isolated strains.

Thermo tolerance

C. sakazakii strains, suspended in TSB and IFM, were determined from 54 to 62°C.At 54°C the values ranged from 15.75 (±0.18) to 18.24 (±0.21) min.

Resistance to low pH

Overall, the mean OD600 of C. sakazakii strains was highest for pH 7.2, which was not significantly ($P > 0.05$) higher than the mean OD600 for pH 5.5. The mean OD600 at pH 4.5 was significantly ($P < 0.05$) lower than the mean\ OD600 at pH 5.5, but difference between OD600 of pH 4.5 and 3.9 was not significant ($P > 0.05$). The isolates were unable to survive at very low pH (2.5)

The growth was maximum at high pH. And thus it was seen that the growth was fine with high pH and there was a very low survival rate at low pH (Healy et al., 2010).

2.1.4 Proteomic analysis of potential virulence factors of *C. sakazakii*

It was found by a series of studies that proteins may provide beneficial information about the virulence of Cronobacter species.in this study the mice was orally injected with Cronobacter image analysis and statistics, protein identification by MS yielded some constant results showing presence of G362 and L3101 .and with the presence of these degeneration of liver and spotty necrosis of liver were seen. OmpA was needed for adhesion and invasion. L3101 caused death within 64h.liver tissues with G362 and L3101

infection showed marked fatty degeneration of liver and necrosis. Also other virulence factors like Dps, OmpA and LuxS was identified in the study which cause infection through adherence and invasion (Ye et al., 2015).

Membrane proteins involved in virulence

There is difference in virulence properties of C. sakazakii G362 and L3101.There was a difference in motility between the two serotypes at 37ºC for 8 hrs.(swimming) and 12hrs(swarming).Swimming ability of L3101 is stronger than that of G362,while the weakest swarming motility was also observed in L3101.

The receptor ompR could influence bacterial biofilm formation and flagella motility by regulating OmpF and OmpC. In this study the flagella motility like swimming motility and swarming motility was determined ,swimming motility of L3101 was stronger, while the swarming was strong for G362 (Ye et al., 2016).

2.2 Biofilm

Bacteria attach to surfaces so that they can develop biofilm. Biofilm consists of a group of microorganisms covered with extracellular matrix. Food-processing environments provide a variety of conditions, which might favour the formation of biofilm, for instance: presence of moisture. The main characteristic of this pathogen is that it forms a biofilm as its shield from pasteurization stresses.

This Biofilm is also an attacking technique into food items to cause infection while escaping the pasteurization stresses. Biofilms are considered to have a heterogeneous structure consisting of micro - Colonies. The biofilm when detaches from the abiotic surface individual microorganisms can easily spread. These organisms that now leave the surface and spread are more resistant to any kind of stresses during food packaging (Brown et al., 1999 and Davey et al., 2000).

The biofilm has been found to form due to the presence of EPS (extracellular polymeric substances).C. sakazakii has been found to have great resistance to desiccation at 25ºC-45ºC than various other species. This resistance to desiccation is brought about by presence of EPS in biofilm Jung et al., (2012) nutrients and inoculum of microorganisms from the raw materials.

2.2.1 Biofilm structure

The confocal scanning laser microscope (CLSM) is used to observe cells responsible for biofilm formation. Biofilm has 15% by volume of microbial cell and 85% matrix material. Backbone of EPS contains 1,3 or 1,4 β linked hexose residues.

There are three main stages in biofilm formation. The amount of EPS is said to increase with the age of biofilm. EPS may associate with divalent cations, metal ions and macro molecules. EPS production is said to be affected by nutrient media, nitrogen content etc. (Kokare et al., 2007).

2.2.2. Biofilm formation procedure:

Firstly, the Free-floating bacteria come in contact with surface and get attached. Production of EPS takes place for bacteria to grow.

Secondly, EPS allows biofilm to develop to a protected sheath.

Thirdly, Biofilms can propagate by leaving the surface and gets established independently to propagate (Jung et al.,2013).

- Initial attachment of microbial cells to the surface,
- permanent attachment,
- Cells proliferation and formation of EPS (maturation I),
- Formation of biofilm structure (maturation II),

• Biofilm spreading (dispersion).

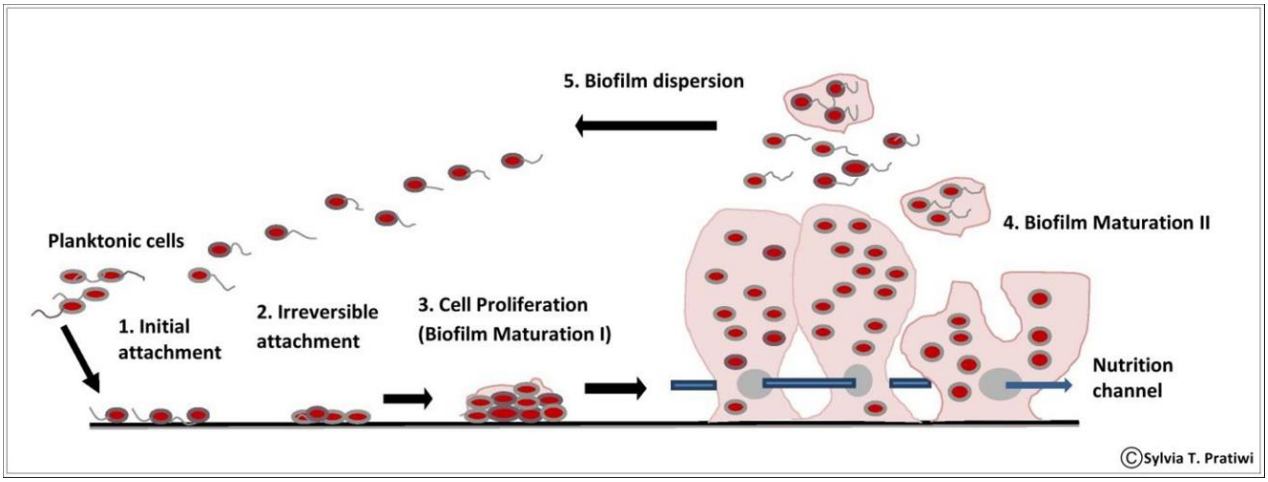

Figure 2: Stages of biofilm formation.

2.2.3. Properties of biofilm

1) Environmental protection.

The EPS plays a major role in structure and function of the biofilm and protects against the environment.it acts as an anion exchanger and prevents exchange of materials from the environment. EPS chelates metal ions, cations and toxins and provide protection from a variety of environmental stresses.

2) Nutrient availability

In this biofilm there is enormous amount of water which helps in transportation. Also biofilm consists of consortia of microorganisms which deal with exchange of materials and excretion of metabolic products. Thus biofilm is also an elite case of syntrophism.

3) Acquisition of new gene target.

For survival of the biofilm for a longer period of time it is necessary for horizontal gene transfer to take place. This occurs by transcription of new genes by biofilm forming communities. This may also in future attribute to some of the antimicrobial properties of biofilm.

4) Penetration of antimicrobial agent

EPS acts as a transport of substances to the interior and their attachment to the surface. All energy is utilized in formation of the EPS for protection against foreign agents.

Solid surface exposed to aqueous medium are more conditioned for growth of biofilm. Biofilm in laminar flow are patchy and rough the ones in turbulent flow are patchy and elongated. Cell to cell signaling is said to play a role in biofilm formation.

Quorum sensing communicates intracellular with gene for biofilm formation. Bacteria rely on small chemical molecules for their cell signaling referred as auto inducers, or acyl homoserine lactones (AHL) (Kokare et al., 2007).

2.2.4. Effects of EPS on biofilm formation

EPS plays an important role in the formation of a biofilm Jung et al., (2012) have described the formation of a biofilm and production of EPS may be affected by various conditions. *C. sakazakii* due to EPS presence enhances the resisting capabilities of the pathogen to desiccation. EPS production may enhance resistance to desiccation in biofilms (Jung et al., 2013).

2.2.5 Biochemical and genetic characteristic of *Cronobacter sakazakii* biofilm formation

In 2012 biofilm forming abilities of 14 C. sakazakii isolates and their mutants was observed. With the use of methods like analysis of biofilm formation capacity, generating transposon mutants, SEM, Raman spectroscopy analysis etc. it was finally confirmed. The biofilm forming capacity of the milk powder from china was maximum the strain being IQCC 10423

Two genes RM2.RM 3 play role in biofilm formation (Du et al., 2012).

2.2.6 Prevention of biofilms

Prevention and control

It was seen that the operations being carried out were the main reason for contamination to occur. Also there was a lot of change due to the sanitation of place. Only if there was a proper cleaning process, use of laminar air flow could this danger be averted. Moreover, nisin-coated PET bottles reduced the production of microbes may be due to nisin of the plate or the nisin being released.

Removal and eradication

Cleaning processes

Its main objective being the removal of biofilm from the surface. The main removal of microbes from the surface is by a chemical method, physical force is applied when it is necessary to remove the microbes from the surface using a mechanical force.

Chemical disinfectants

Chemical disinfection is very useful in case of surface active microbes (i) chlorine-based compounds, hydrogen peroxide, ozone and PAA, (ii) quaternary ammonium compounds and acid anionic compounds (iii) iodophores, pH, temperature, concentration, contact time and interfering organic substances like food particles and dirt influence disinfection. Also quorum sensing is being explored.

Physical methods

With the use of active plasma inactivation a lot of removal could obtain massive cleaning. Ultrasound, UV treatment, enzyme technology and temperature could immensely reduce the microbes in the environment, milk. Also electric fields act as essential biocides in the process.

Therefore to avoid the biofilm from households, industries, hospitals etc. we can

•Develop cleaning plan and disinfection programs.

•Include biofilm supporting properties like their thermal resistance etc. as a hygienic design

•Identify biofilm prone areas and prevent infection

•Develop better plans for researching the capability of disinfectants (Houdt et al., 2010).

2.3 Inhibition of biofilm formation by *C. sakazakii*

Knowing the amount of havoc biofilms can cause by shielding the *C. sakazakii* during its stages of infection.it has become a serious issue on how to deal with the biofilm and inhibit its activity so that it does not hamper its functions in the households, hospitals, food industry etc.

2.3.1 Biofilm under modified atmospheric conditions

It was demonstrated that the effect of varying atmospheric conditions on the biofilm. Although there was no proper inhibition in biofilm formation but there was decrease in their formation. This study aimed at determining survival characteristics of biofilm cells of *C. sakazakii* as affected by different atmosphere conditions. Survival of cells in biofilms formed on stainless steel and PVC on exposure to air, N2 gas, and CO2 gas for up to 20 days was determined.

N2 gas resulted reductions of 0.98, 1.34, 1.96, and 1.79 log CFU/cm2 after 5, 10, 15, and 20 days of storage, respectively, compared to air storage. CO2 gas reductions of 1.31, 1.45, 2.04, and 2.47 log CFU/cm2 after 5, 10, 15, and 20 days of storage, respectively, compared to air storage. N2 and CO2 gases led to less reduction of biofilm cells on PVC compared to those on stainless steel. The initial

Biofilm cells of C. sakazakii on PVC were 7.09 log CFU/cm2. N2 and CO2 gases resulted in significant reductions of 0.98 and 1.20 logs CFU/cm2 after 20 days of storage, respectively, compared to air storage

Reduction was more under nitrogen and carbon dioxide than the air (Park et al., 2014).

2.3.2 Effect of calcium and magnesium on the biofilm formation

In 2015 it was demonstrated that the effect of calcium and magnesium on the biofilm formation as by then only little information of the effects of ions was available to us. The biofilm-forming ability of 23 *C.*

sakazakii strains was assessed at different $MgCl_2$ (0.00, 0.25, 0.50, 1.00 and 1.50%, w/v) and CaCl2 (0.00, 0.25, 0.50, 1.00 and 1.50%, w/v).the TSB was infused with divalent action by adding (0.50% NaCl-pH 7).

Biofilm was observed by confocal laser scanning microscope (CLSM).the biofilm was inhibited by MgCl2 concentration from 0.25% to 1.50%.CaCl2 also inhibited the biofilm formation on increase of concentrations. The OD ranged from 0.434 to 3.277 at 0% to 0.553 to 3.685 at 1.50% MgCl2.0.434-3.277 at 0% to .326-2.306 at 1.50% CaCl2. Divalent cation is said to be affecting adhesion properties.at 1.50% of MgCl2 and at 0.5% of CaCl2 highest biofilm was formed (Ye et al., 2015).

2.3.3. Effects of milk components and probiotic LAB cultures in inhibiting biofilm

2.3.3.1. Influence of copper sulphate, lactic acid, monolaurin and milk components

Copper (II), monolaurin and lactic acid act as natural antimicrobial agents. Lactic acid not only increases the shelf life of food but also is easily accessible. Copper at high concentrations, causes the inhibition of growth in bacteria, fatty acids enhances antimicrobial activity against certain pathogens.

Monolaurin exhibited antibacterial activity against Listeria monocytogenes and against Escherichia coli O157:H7 in UHT milk. A complete elimination of pathogen was seen at LA (0.2%) and copper (II) (50µg/ml) was used.

A complete elimination of pathogen was seen when combination of sub lethal concentration of LA (0.2%) and copper (II) (50µg/ml) was used. 6.70 log10 as compared to 7.56 log10 copper (II) and LA at 100µg/ml copper (II) Highest antimicrobial activity was observed at 0.2%LA and 100µg/ml copper (II) complete eradication was observed (Holy et al., 2010).

A few scientists wanted to determine the critical components in skim milk for biofilm formation. There was a wide variation in biofilm forming ability

In conclusion, this study provides evidences that, for biofilm formation of Cronobacter species in skim milk, nitrogen source is probably a more important determinant than carbohydrate, and indicates that Cronobacter strains have a wide variation in biofilm forming ability The OD at 570 was skim milk>1:8W>1:8C>1:8L>1:8diluted skim milk. Seeming nitrogen to be an important determinant than carbohydrate (Dancer et al., 2009).

2.3.3.2 Influence of probiotic cultures on biofilm

The effect of probiotic cultures was investigated on the antibacterial activity of *Lactobacillus acidophilus* and *L. casei* isolated from feces of healthy infants against different strains of *C. sakazakii*. probiotics (LAB have GRAS status) inhibit the growth of different types of foodborne pathogens..

LAB isolates including *L. casei, L. acidophilus, L. johnsonii, L. reuteri, L. rhamnosus, and Bifidobacterium spp*. against a wide range of foodborne pathogens expressed antimicrobial activity. Such actions of *L. casei and L. acidophilus* against *C. sakazakii* may occur naturally in the gastrointestinal tract. In this study both were isolated from infant feces. LAB culture was maintained in 20% glycerol stock and six strains of Cronobacter were isolated from PIF after screening of LAB for antibacterial activity, inhibitory functions they were treated with Cronobacter species.

Both the lactobacillus cultures inhibited growth of 2 strains of Cronobacter. The inhibitory effect was mainly due to the production of bacteriocins. Also the LAB helped in stabilizing gut environment.

in infant formula probiotics usually act towards a healthy gut microbiota development (Collado et al.,2008).

2.3.3 Influence of kefir on biofilm.

Kefir produced by the microbial activity of kefir ‘‘grains’’ (starter cultures), which have a relatively stable and specific balance of lactic and acetic acid bacteria and yeast. is a type of fermented milk originating from the Caucasus Mountains. Many pathogenic bacteria, including *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Escherichia coli, Salmonella, Shigella flexneri, Shigella sonnei,* and *Pseudomonas aeruginosa* are inhibited by the antimicrobial activity of kefir. The growth of all 20 *C. sakazakii* strains was completely inhibited by the addition of kefir supernatant, as determined by the spot-on-lawn test.

This is the first study demonstrating the antimicrobial activity of kefir against *C. sakazakii*. There were no differences between clinical and food isolates in terms of the degree of inhibition by kefir. In control

cultures containing 100µl of milk supernatant, 2.1×109 CFU/ml C. sakazakii grew on the nutrient agar. In contrast, in cultures containing 100, 150, or 200 µl of kefir supernatant, there was no growth of C. sakazakii on the agar, indicating a sterilizing effect in the 24-h growth test on the nutrient agar.

It has been consumed for centuries, and therefore, no harmful health effects are expected from consuming kefir supplemented PIF. Second, infants are at the highest risk of *C. sakazakii* infection but are also vulnerable to the side effects associated with antibiotics (Kim et al., 2015).

2.3.4 Influence of polyphenols on biofilm

2.3.4.1 Effect of natural antimicrobial substances in red muscadine juice

Muscadine grapes tolerate high hot, humid climate and also resist the pierce's disease. They are known for their antioxidant activity, anti-inflammatory and ant cancerous property .Their juice is good in natural organic acids, phenolic acids, and thus may have antimicrobial properties against *C. sakazakii* of baby food.

After preparation of juice samples and the determination of polyphenol content and quantification of polyphenolic compounds it was seen that tartaric acid, gallic acid, catechins, epicatechin was found in abundance. Antimicrobial activity was seen in which within 2h both strains of C.sakazakii was reduced to non-detectable levels. Antimicrobial activity was seen in which within 2h both strains of C.sakazakii was reduced to non-detectable levels.

Tannic acid in muscadine juice showed the highest inhibition. Approximately a 4 log reduction was seen within 2hrs.Tannic acid in muscadine juice showed the highest inhibition. Approximately a 4 log reduction was seen within 2hrs (Kim et al., 2010).

2.3.4.2 Effect of blueberry procyanidins

Blueberries are known to exhibit strong antioxidant capacity associated with phenolic acids, catechins (flavanols), and proanthocyanidins (condensed tannins). The antimicrobial activity against *Listeria*

monocytogenes, Salmonella Typhimurium, Escherichia coli and Candida albicans therefore they used blueberry extract to reduce the effect of *C. sakazakii* strains.

There was reduction in the growth on treatment with blueberry procyanidins and this as a whole reduced within an interval of 7 hrs. from treatment 8.255 log CFU/ml after 1h with 5mg/ml blueberry PAC and BJ.after 3h and 6h >8.58 reduction was seen. Malic acid at pH of 3 led to reduction of 1.2 log CFU/ml after 6 hr. 5 log cells were inactivated in 1.5 hrs. From treatment 8.255 log CFU/ml after 1h with 5mg/ml BJ.

After 3h and 6h >8.58 reduction was seen. Malic acid at pH of 3 led to reduction of 1.2 log CFU/ml after 6 hr. Prominent blebs were seen on treatment with BJ showing cell disruption. These features could play a role in biofilm formation protecting the cells from antibacterial effects of BJ (Joshi et al., 2014)

2.3.4.3 Effect of trans-cinnamaldehyde

Trans-cinnamaldehyde is the major component of bark extract of cinnamon. It was seen

That TC could inactivate PIF. TC was found to modulate genes associated with biofilm synthesis. In a crux TC would inhibit biofilm formation on polystyrene microtiter plates, stainless Steel, enteral feeding tubes.*C. Sakazakii* was helpful against biofilm in infant formula. bcsA, bcsG, fliD, flhD, flgJ, motA, motB, luxR genes play a role in biofilm formation.it was seen that TC down regulated their expression.TC could be used as an antimicrobial to prevent and inactivate biofilm.

The TSB and IFB biofilm associated population was reduced by 4 and 3 log CFU/ml after 96h in well with 750µM TC from an initial 7.5 log CFU/ml. experiments performed in enteral feeding tubes, stainless steel also showed a considerable reduction. bcsA, bcsG, fliD, flhD, flgJ, motA, motB, luxR genes play a role in biofilm formation.it was seen that TC down regulated their expression (Amalaradjou et al.,2011).

2.3.4.4. Effect of tea polyphenols

It was investigated that the effect of tea polyphenols on the 4 C. sakazakii strains isolated from PIF. The various polyphenols like catechins, phenolic acids, flavonoids, anthocyanin exhibit anti-cancerous etc.

properties. The PIF was treated with organic and inorganic acids both and turbidity measured simultaneously. After 7 h treatment, the final pH of samples containing 5 mg/mL malic acid, citric acid, VC and TP were 3.64, 3.58, 4.57, and 6.49, respectively.so after 7 h pH were almost stabilized. The pH decreased 3.62, 3.55, and 4.55 respectively.

The order of inhibition was citric acid (3.55)>malic acid (3.62)>VC (4.55)>TP6.46)>PIF(6.82).TP becomes an efficient antibiotic. The TP that was acidified with HCl had totally inhibited sakazakii after 7h.the antimicrobial effect of the acids was increased after their acidification. After 7 h treatment, the final pH of samples containing 5 mg/mL (3.44) malic acid, citric acid, VC and TP were 3.64, 3.58, 4.57, and 6.49, respectively.so after 7 h pH were almost stabilized.

The TP that was acidified with HCl had totally inhibited sakazakii after 7h.the antimicrobial effect of the acids was increased after their acidification. Cells treated with TP were seriously damaged and plasma leaked out thus the effect being bactericidal (Li et al., 2016).

On the contrary, Lactic acid bacteria (LAB) play an important role in different food fermentations in the form of starters. This group of bacteria is generally recognized as safe (GRAS microorganisms). The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (Daschle, 1989), various antimicrobial compounds, such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2, 3-butanedione), D-isomers of amino acids, reuterin and bacteriocins (Cintas et al. 2001).

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals Requirements:

Soya bean casein digest medium, Enterobacter sakazakii hi veg agar modified, hi media agar, *C.sakazakii* cultures (N 112, N13, N15, E604,CS), glycerol, distilled water, absolute ethanol, methanol, crystal violet, glacial acetic acid, tris HCl, EDTA, NaCl, SDS, chloroform, isopropanol, agarose,, MRS, ammonium sulphate, SDS, acrylamide, bus-acrylamide, TEMED, Ammonium Per sulfate, beta mercapethanol, bromophenol blue, glycerol, glycine and PBS buffer, DMSO, glacial acetic acid, methanol, coomasive dye.

3.1.2 Other Requirements

Petriplates, test tubes, inolculation loop, Bunsen burner, coverslip, micropipette, 12 well microtiter plate, microscope, ependorfs, centrifuge, microwave, gel tray, well comb, buffer tank, gel doc, PCR vials, tarson tube, syringe filters, syringe, spreader, well borer, 96 well plate, magnetic stirrer, magnetic beads, dialysis bag, thread, a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs and glass plates.

3.2 Methods

3.2.1 Mantainence of *Cronobacter sakazakii* and lactic acid bacteria cultures

The cultures N112, N13, N15, E604, CS were available in the lab i.e.

1) CS (ATCC 12868)
2) E604
3) N13 fruit
4) N112 fruit

5) N15 soil

These were activated from the glycerol stock in tryptic soya broth (TSB) or soyabean casein digest medium and incubated at 37ºC overnight.The cultures were streaked onto Tryptic soya agar (TSA) and Enterobacter sakazakii agar (ESA) plates and incubated overnight. ESA showed bluish-green colonies on the plate. Isolated colonies from each strain were inoculated in 80% glycerol stock and stored. The lactic acid bacteria cultures were isolated from different fermented foods of himachal Pradesh already in the lab i.e. C1, 4, 7, 14, 29, 90, 94, 98, LRGG).

1) *Lactobacillus rhamnosus* (from NDRI Karnal)
2) *Lactobacillus paracasei* (CD4)
3) *Lactobacillus gastricus* (BTM 7)
4) *Weisella confuse (CD 1)*
5) *Lactobacillus* Sp. 90
6) *Lactobacillus* Sp. 94
7) *Lactobacillus* Sp. 98
8) *Lactobacillus* Sp. 14
9) *Lactobacillus* Sp. 29

The cultures were inoculated in MRS broth and left at shaking at 120 rpm at 37ºC overnight. The next morning the lactic acid cultures were centrifuged and supernatant retained and filtered using syringe filters.

3.2.2 A 96-well plate based MIC (minimum inhibitory concentration) test for probiotic culture

TSA tubes were inoculated with pathogen and incubated overnight. In the microtiter plate 200µl fresh TSB was added .After which 10µl of culture of pathogen was added. And lastly 10µl of different probiotic cultures were added (C1, 4, 7, 14, 29, 90, 94, 98, 100, LRGG). After incubation for at least 6 hours the result was observed in the 96 well Elisa plate reader.

3.2.3 Zone of inhibition test by agar well assay method for probiotic cultures

18 strains of lactobacillus species isolated from different probiotic foods were procured from the lab and grown in MRS broth and incubated overnight (4, 9, 7, 13, 14, 29, 34, C1, 75, 78, LRGG, 100, 84, 90, 98, 94). In TSB the 5 strains of Cronobacter were activated (CS, N112, N13, E604, N15).After the growth in MRS the MRS Tarsons were centrifuged at 15000 rpm for 30 minutes. The pellet was discarded and the supernatant was filter sterilized using syringe filters. The pathogen strains were spread on the TSA plates (a 100µl of each Cronobacter culture was spread) using the spreader wells were dug into the borer using a well borer. The cultures were filled in the wells (100µl of probiotic cultures) and incubated overnight to observe the zone of diffusion.

3.2.4 A 96 well based MIC (minimum inhibitory concentration) test for trans-cinnamaldehyde

CS and E604 were activated a night before.200µl of TSB was added to the plate.5µl of pathogenic samples were added. Extracts of trans-cinnamaldehyde were added in the well in the quantity (1, 2.5, 5, 7.5, 10µl).The plates were incubated overnight and readings taken in 96 well ELISA plate reader.

3.2.5 Zone of inhibition test by agar well assay method for trans-cinnamaldehyde

This test was performed as a control to check the efficiency of probiotics as antimicrobial agents. In TSB the 5 strains of Cronobacter were activated (CS, E604). A 100µL of culture was spread on the plate with agar and 5mm wells were bored to which different dilutions of 50µl trans-cinnamaldehyde were added from 1:10,1:20,1:30,1:40,1:50. The plates were incubated at 37ºC and readings taken after 6hrs by measuring the diameter of the zone.

3.2.6 Ammonium sulfate precipitation

For ammonium sulfate precipitation a table is seen and the volume of broth that needs to be precipitated tells us the amount of ammonium sulfate that has to be added. Different cuts for 0-40%, 40-60%, 60-80% and 80-100% were made according to which the amount of ammonium sulfate to be added was decided. The samples 90, 98 were subjected to the precipitation. First 20 ml of the sample was kept in a small beaker around which ice was kept, according to s all the pellets were stored at 500µl PBS.

3.2.7 Dialysis

Dialysis bags were obtained. One end of the bags was tightly tied with thread and the precipitated sample was poured from the other end which was again tied tightly. The bags were immersed completely in PBS buffer solution for osmosis to take place. The PBS buffer which was a hypotonic solution was changed from time to time to allow the removal of smaller particles that had travelled across the membrane. This assembly was kept in 4º with magnetic beads in it to help in circulation of media.

Later the protein content was estimated using the

3.2.7.1 Lowry method

Lowry A – 2% sodium carbonate in 0.1 N sodium hydroxide

Lowry B – 1% copper sulphate in distilled water (100ml)

Lowry C – 2% sodium potassium tartrate

Lowry solution- 49ml A + 0.5ml B + 0.5ml C

Folin solution (2N) t0 distilled water (1:1)

BSA standard was prepared (1 mg/ml) dilutions were made 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard was pipeted into the tubes. 10 µl of culture was added to test tube to which 5 ml of Lowry solution was added and vortexed for 15 minutes. 0.5 ml of folin reagent was added and incubated for 30 min with O.D. measured at 750 nm.

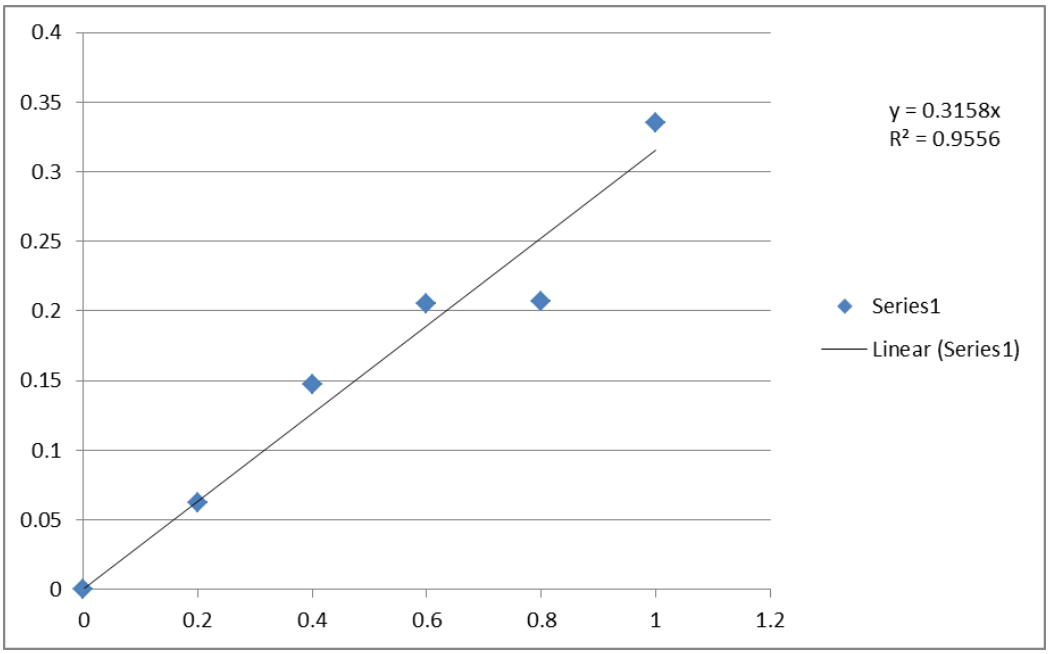

Figure 3 : BSA standard

3.2.8 Zone of inhibition test by agar well diffusion assay for bacteriocins of probiotic cultures

The pathogen culture for CS and E604 was activated. The 100µl of culture of the two strains was spread on the TSA plates. Wells were made on the plates of 5mm diameter and 50µl of the bacteriocins cultures 90,98 that showed the best results were taken i.e. 90(0-40%, 40-60%), 98(0-40%).The plates were incubated for 6 hrs. and then the diameter was measured for zone of inhibition.

3.2.9 SDS-PAGE

M Tris- pH=8.8

Dissolved 18.16g of Tris base in 100ml distilled water, for 200 ml 36.38g of Tris base. pH adjusted to 8.8 by using concentrated HCL (should be handled carefully). Stored at 4°C.

1M Tris- pH=6.8

Dissolved 12.11g Tris base in 100ml of distilled water and pH adjusted to 6.8 using concentrated HCL. Stored at 4°C.

10% Ammonium Persulphate (APS)

Dissolved 100 mg of APS in 1 ml of Distilled water. Solution must be prepared fresh every time.

30% Acrylamide

Prepared in Dark bottle and stored at 4°C.

Table1:30% acrylamide

Component Vol.	**25ml**	**50ml**	**100ml**
Acrylamide	**7.5g**	**15g**	**30 g**
Bis-acrylamide	**0.2g**	**0.4g**	**0.8g**

Sample buffer

Mixed all the components in distilled water and raised the volume up to 10 ml.

Components	amount
Tris HCl (pH 6.8)	1.25 ml
Glycerol	2 ml
Bromophenol blue	pinch (0.01 mg)
SDS	0.4ml
β Mercapethanol	0.5ml (fresh)

Components of Running buffer (10x)

Mixed all the components below in distilled water and raised the volume up to 1L with distilled water. pH of this solution must be between 8.3

Components	Weight (g)
Tris base	30.3
Glycine	144
SDS	10

Components of Resolving Gel- 12%

Table 2: Resolving gel 12%

Total volume	**10ml**
Distilled water	**3.3ml**
30%acrylamide	**4ml**
1.5 Tris (pH-8.8)	**2.503**
10%SDS	**100µl**
10%APS	**100µl**
TEMED	**4µl**

Components of Stacking Gel- 5%

Table 3: stacking gel 5%

Components vol	**5ml**
Distilled water	**3.4**
30% acrylamide	**830µl**
1 M Tris(pH-6.8)	**630**
10% SDS	**50µl**
10% APS	**50 µl**
TEMED	**5 µl**

Staining solution- 500ml

Components	Vol. (ml)
Methanol	400
Distilled water	500
Glacial acetic acid	100
Coomasive R250 dye	0.5g

Destaining Solution- 1000ml

Components	Volume (ml)
Glacial acetic acid	100
Methanol	400
Distilled water	500

PROCEDURE

Washed the glass plates, rubber stopper, comb with distilled water, dried them at 45°C and wiped them with absolute ethanol. The casting frame was settled between the clamps. To check for the leakage distilled water was added and removed subsequently. 12% resolving gel was prepared and about 5 ml was pipetted between the plates. It was then left to gelate and to check for the leakage 1 ml of water was added which was removed once the gel solidified. After the solidification of this gel 5% stacking gel was made and it was kept in mind that it had to be pipetted as soon as TEMED was added. The gel was pipetted in till the time it overflowed, soon after this the comb was inserted tightly and left till the gelation of the well.

The comb was removed after the gel was set and the clamp was released so that the gel in the plates could be placed in the buffer dam Poured 1 x running buffer (electrophoresis buffer) into the inner chamber.

Preparation of samples: Mixed 10µl sampler with 10µl of sample buffer and denatured it for 10 min at 95ºC. The protein ladder was loaded in the first well followed by 18 µl of samples in the subsequent wells. Running buffer was poured between the two gel plates and after all checks electrophoresis was started at 100V. The SDS-PAGE was stopped once the blue dye reached the foot of the glass plate. After this the gel was placed in a plastic box filled with staining solution and left on the rocker at 10 rpm speed overnight. After this the gel was immersed in destaining solution for intervals of 1 hr. during which fresh destaining solution was being continuously added and the old one removed. Next the bands were visualized and results confirmed.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Maintenance of cultures

The culture of *C. sakazakii* was grown in TSB and then plated onto the TSA and ESA plates. ESA is a chromogenic media that provides bluish green color to the pathogen. The selected colonies were then taken an d used for experiments.

4.2 MIC by Lactic cultures

The lactic cultures were used so that their efficiency of inhibiting the pathogenic *C.sakazakii* could be tested. It was seen that maximum inhibition was seen in a range of 10 – 50 µl of each CFS of probiotic cultures. Whereas, 50 µl of the supernatant provided the most amount of inhibition i.e. upto an O.D. of 0.083. Out of all the samples C1, 7, 9, 90, 94, 98 showed the lowest growth of the pathogen.

Table 4: MIC values for C.sakazakii ATCC 12868 in different *Lactobacillus* cultures

LAB Strains	Broth	Broth+ culture (CS)	Broth +10µl LAB supernatant	Broth +10µl LAB supernatant +culture(CS)	Broth +25µl LAB supernatant	Broth +25µl LAB supernatant +culture(CS)	Broth +50µl LAB supernatant	Broth +50µl LAB Supernatant +culture(CS)
C1	0.044	0.473	0.082	0.268	0.050	0.153	0.058	0.086
7	0.045	0.477	0.075	0.235	0.052	0.141	0.068	0.098
9	0.045	0.468	0.069	0.221	0.059	0.150	0.056	0.085
14	0.045	0.478	0.066	0.257	0.060	0.170	0.055	0.100
29	0.044	0.460	0.073	0.202	0.051	0.141	0.055	0.097
90	0.043	0.478	0.076	0.204	0.048	0.140	0.057	0.098
94	0.045	0.480	0.078	0.184	0.057	0.143	0.061	0.087
98	0.045	0.462	0.081	0.188	0.057	0.148	0.054	0.083

Out of all the probiotic cultures the above mentioned few showed consistent good results against the C.S. strain of the pathogen throughout.

It was seen that the culture 90 showed the maximum percentile (61.65% - 84.85%) of inhibition in 10 - 50µl supernatant. This showed the presence of inhibitory components in the 90th strain. There was no inhibitory activity of lactic acid bacteria against other C.sakazakii isolates therefore further studies were conducted on only C.sakazakii ATCC 12868.

Table 5: % inhibition of lactic cultures against *C.sakazakii* ATCC12868

Strains	**% inhibition with 10µl CFS**	**% inhibition with 25µl CFS**	**% Inhibition with 50µl CFS**
***Lactobacillus* Sp. C1**	**46.7%**	**75.23%**	**81.94%**
***Lactobacillus* Sp. 7**	**42.9%**	**64.56%**	**75.99%**
***Lactobacillus* Sp. 98**	**57.65%**	**77.41%**	**85.97%**
***Lactobacillus* Sp. 94**	**59.95%**	**77.56%**	**84.11%**
***Lactobacillus* sp. 90**	**61.65%**	**78.15%**	**84.85%**

.The results obtained on % inhibition of C.sakazakii indicated that the inhibition is strain specific as all other C.sakazakii stains were not inhibited by the CFS of lactic acid bacteria.After the agar well diffusion of the LAB cultures against the pathogen it was seen that lactobacillus Sp. 90 showed a zone of inhibition almost against every strain of the pathogen. The diameter of the zone reached maximum of 20mm.

Table 6: zone of inhibition by lactic cultures against different strains of *C. sakazakii*

Strains	**CS**	**N112**	**N13**	**N15**	**E604**
***Lactobacillus* Sp. C1**	**—**	**—**	**—**	**10mm**	**—**
***Lactobacillus* Sp. 7**	**5mm**	**—**	**—**	**—**	**—**
***Lactobacillus* spp.98**	**15mm**	**—**	**—**	**—**	**—**
***Lactobacillus* spp.94**	**18mm**	**—**	**14mm**	**—**	**12mm**
***Lactobacillus* spp. 90**	**20mm**	**17mm**	**10mm**	**7mm**	**—**

In the agar well diffusion assay, the Lactic isolates 90 and 94 resulted in maximum inhibition of C.sakazakii ATCC 12868 whereas the other C.sakazakii strains were not inhibited by lactic starters. The Lactobacillus strain 90 possessed maximum activity against four strains of C.sakazakii and lactic isolate 94 possessed activity against three strains of C.sakazakii. The results indicated that the inhibitory components in supernatant has strain specific activities.

4.3 Zone of inhibition by agar well diffusion assay using probiotic cultures

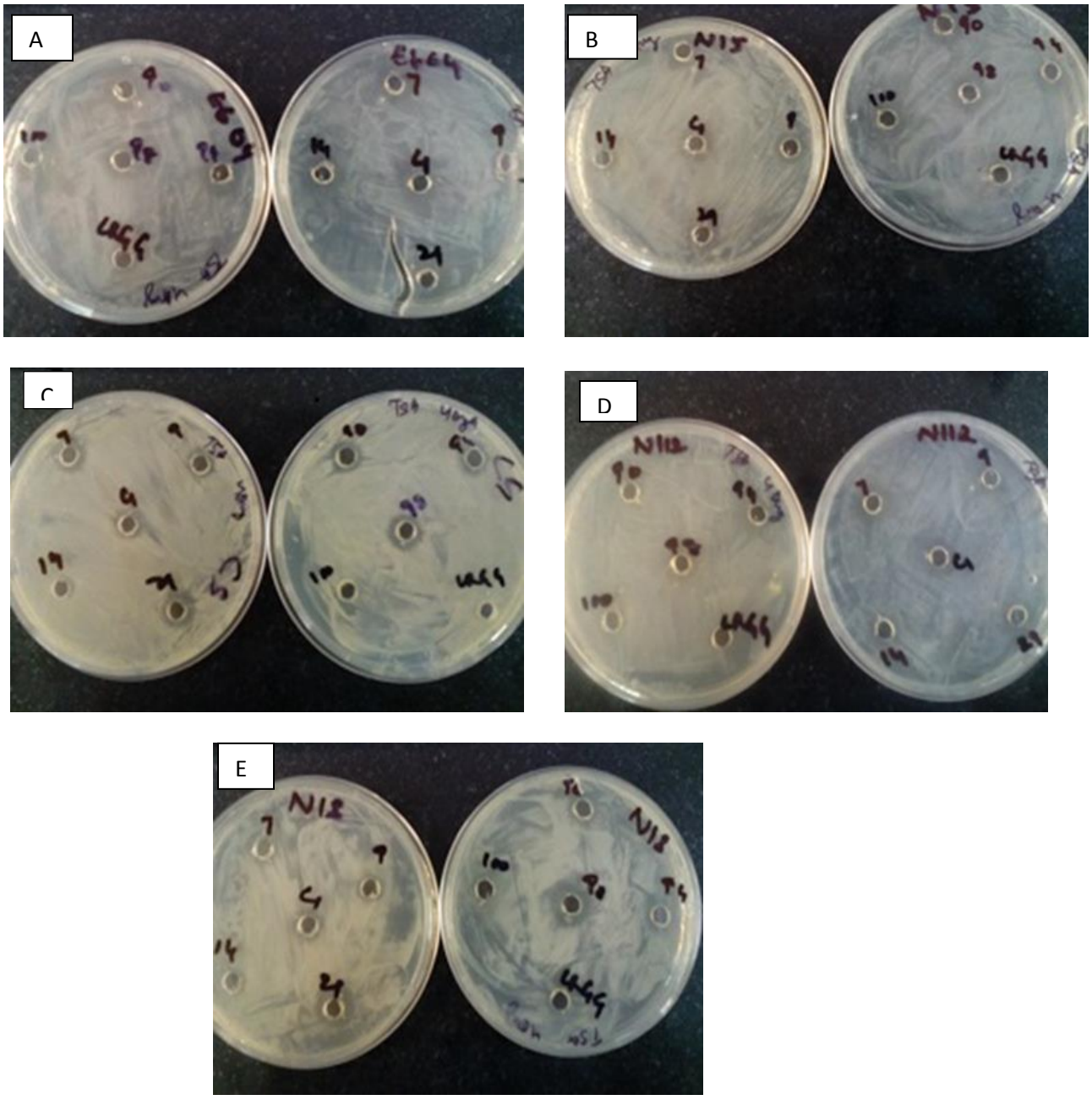

Figure 4: Zone of inhibition seen when probiotic cultures C1, 4, 13, 29, 90, 94, 98, 7, 9, 100, LRGG were used to treat Cronobacter strains A) E604, B) N15, C) CS, D) N112, E) N13

The zone of inhibition was see clearly for the sample C1, 29, 90, 94, 98 mainly against all the pathogenic strains i.e. CS, E604, N13, N11, N15.

When tested against various probiotic cultures the best results were observed from lactobacillus sp.94 and 98 which inhibited the growth of the bacteria to minimal zero levels. Also they produced a zone of inhibition with diameter above 14mm for almost all pathogenic strains.

4.4 MIC of Trans-cinnamaldehyde

Table 7: MIC: % reduction in growth of pathogen as observed under different dilutions of Trans-cinnamaldehyde

strains	**1:10**	**1:20**	**1:30**	**1:40**	**1:50**
CS	75.15%	61.30%	57.40%	47.40%	12.93%
E604	69.33%	55.85%	36.91%	29.29%	18.35%

Trans-cinnamaldehyde when diluted (1:10, 1:20, 1:30, 1:40, 1:50) in DMSO upto various levels inhibited the pathogen with a % reduction ranging from 12.93% - 75.15% against E604, CS. These results were observed after an interval of 6 hrs.

4.5 Antimicrobial activity of trans-cinnamaldehyde

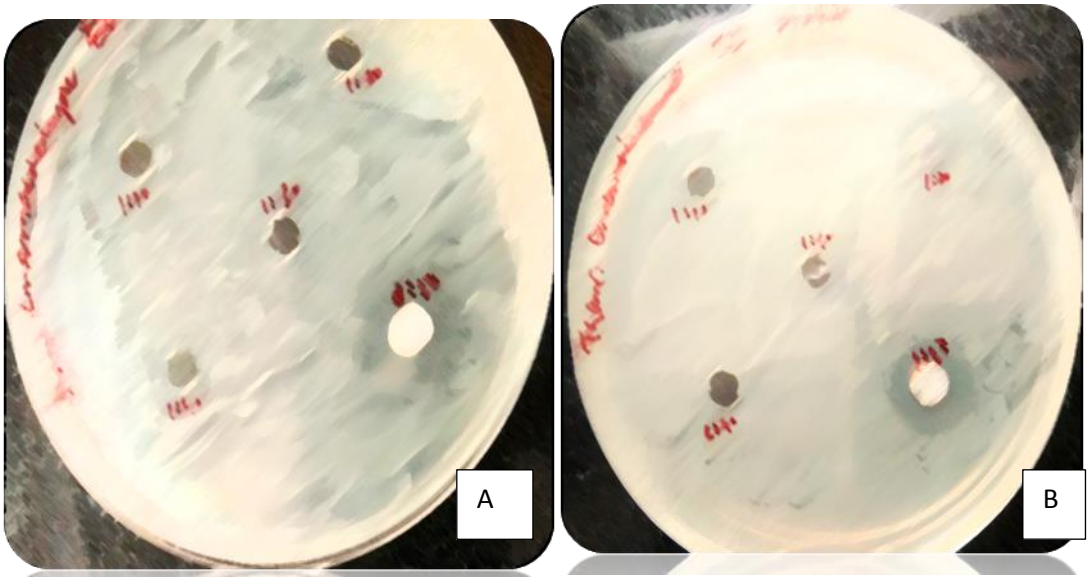

Figure 5: A) E604 showed zone of inhibition against dilution 1:10 and B) CS showed zone of inhibition for 1:10 & 1:30 dilution of trans- cinnamaldehyde

The antimicrobial activity of trans-cinnamaldehyde was compared with that of probiotic cultures and it was seen that probiotic cultures had a far better effect on the antimicrobial activity of the pathogen . as trans cinnamaldehyde could only restrict the growth upto 75% during MIC and only 1:10 dilution of the compound showed a zone of inhibition and that too was not distinct

4.6 Dialysis

Dialysis was performed using the dialysis bag this was done to purify the CFS after the ammonium sulphate precipitation. The ends of the bag were tied with a thread and the entire assembly were kept in PBS in ice cold conditions for salting out of salts to take place. After the interval of 12 hrs pure protein was produced.

Figure 6: Dialysis in dialysis membrane bag

4.7 Estimation of protein content and antimicrobial activity against C.sakazakii ATCC 12868

Table 8 : protein estimation

% saturation	protein content (mg/ml)	zone of inhibition
CFS of Lactobacillus sp. 90	2.67	15mm
0 – 40%	0.420	10mm
40 – 60	0.450	_
60 – 80	0.370	_
80 -100	0.450	_

4.8 Agar well diffusion assay of dialyzed samples 90

After the ammonium sulphate precipitation of the probiotic cultures and dialysis the bacteriocins was checked for the antimicrobial activity by performing the agar well diffusion assay and 0-40% saturation for the 90 sample showed effective antimicrobial results against the standard E604 strain.

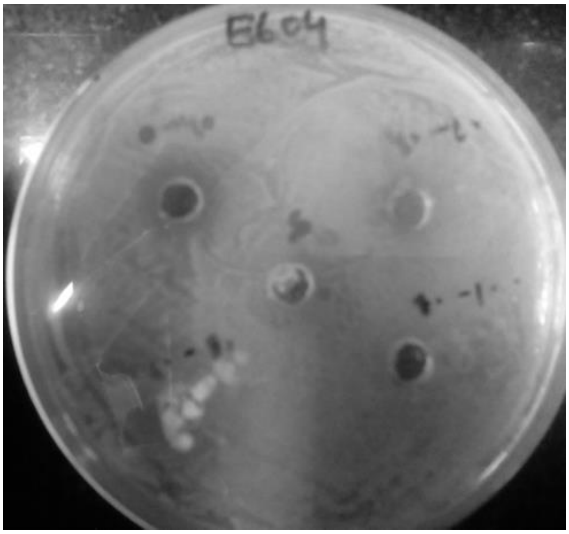

Figure 7: Zone of inhibition was seen for bacteriocins obtained from 0-40% of ammonium sulphate precipitated strain of lactobacillus spp. 90 against E604 strain of pathogen *C.sakzakii*

4.9 SDS PAGE

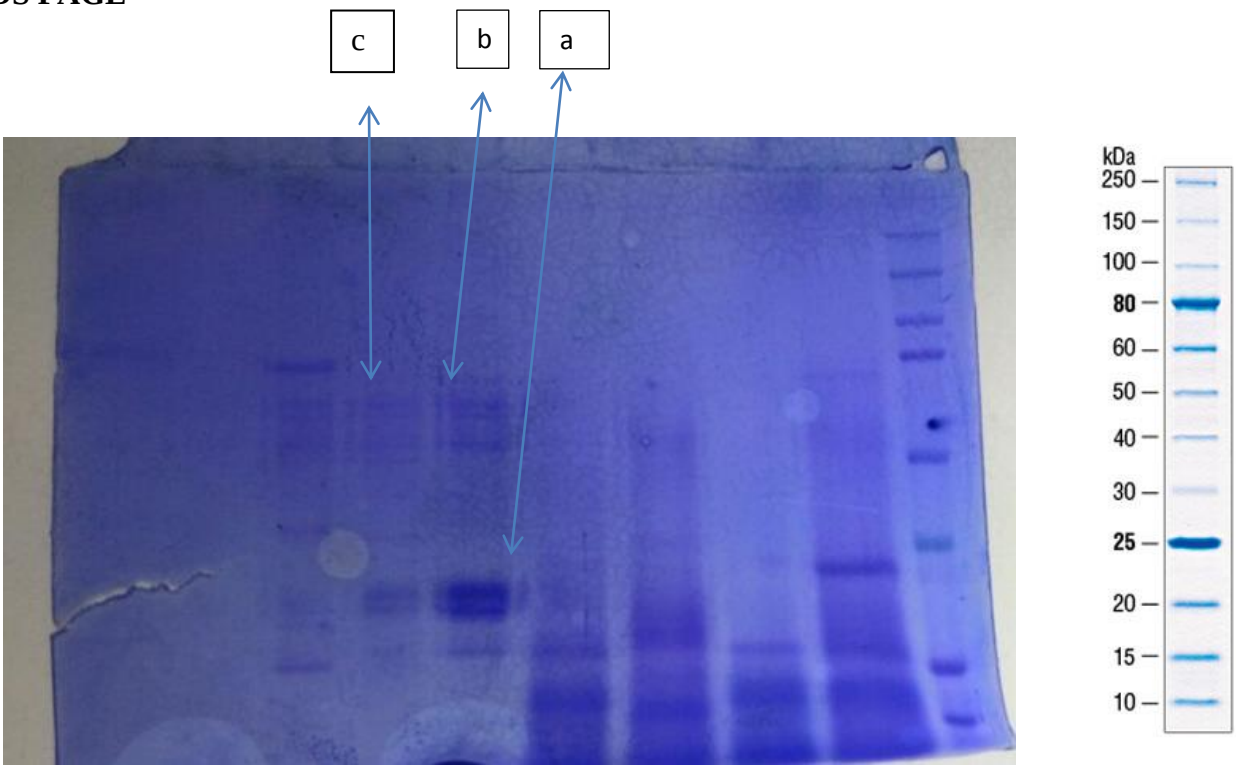

Figure 8: SDS PAGE results for a) 90 (0-40%), b) 90 (40-60%), c) 98 (0-40%)

A smear was seen for 0-40% in 90th sample confirming the presence of bacteriocins in it. Although 98 sample also showed faint bands but no considerable result was seen in its case in agar well diffusion assay. The smear in the 90 was a partial proof of presence of proteins and that the bacteriocins were responsible for antimicrobial activity.

This report dealt with the antibacterial activity of the probiotic cultures. These cultures were isolated from the fermented foods of Himachal Pradesh like bhaturoo, milk, curd etc . The cultures were already available in the lab. The pathogenic *C.sakazakii* was isolated from different samples like rain water, fruit, beverage etc. this was also available in the lab.

When the MIC was performed with the LAB cultures available in the lab maximum inhibitory reduction of 85% was seen for a volume of 50µl of the probiotic CFS after an incubation time of 6hrs., the zone of inhibition in the above case ranged from 7mm to 17mm.Thus, proving the antibacterial activity of LAB.

Awaisheh et al. (2013) had previously evaluated the antibacterial activity of pH neutralized CFS by *L. casei* and *L. acidophilus.* They had suggested the presence of bacteriocins as the probable cause of the activity. To further investigate the reason for antibacterial activity i.e. pH, heat or bacteriocins ammonium sulphate precipitation was performed followed by dialysis for purification of the sample. The CFS generated was plated against the standard pathogenic cultures CS and E604. The LAB culture taken was 90^{th} as it showed best results for all the 5 pathogenic strains. At 0-40% saturation cut E604 was inhibited with a 9mm zone of inhibition. The presence of protein was confirmed by bands in SDS.

The TSB and IFB biofilm associated population was reduced by 4 and 3 log CFU/ml after 96h in well with 750µM TC from an initial 7.5 log CFU/ml (Amalaradjou et al.,2011). Trans-cinnamaldehyde activity was also studied in the lab just to compare the inhibition of both the compounds i.e. CFS and trans-cinnamaldehyde. The maximum MIC trans - cinnamaldehyde showed was 75% as opposed to 84.5% by CFS. And also it showed the zone of inhibition at 1:10 dilution only.

Therefore it was observed that the LAB was more potential in exhibiting anti-inhibitory activity but the exact component was not determined.

CHAPTER 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Probiotics exhibit numerous benefits ranging from nutritional benefits to maintaining healthy gut environment. In the present study additional benefit of probiotic cultures from fermented foods of Himachal Pradesh were explored. The Lactic Acid Bacteria were isolated from different foods of Himachal like bhaturoo, milk, curd etc. all these strains were tested for their antimicrobial activity against *Cronobacter sakazakii*. . It was seen that about 44-84% inhibition was observed when C.sakazakii was treated with 10-50µl of different LAB cultures . The agar well diffusion assay gave a zone of 14-18mm for cultures *Lactobacillus* Sp. 90, *Lactobacillus* Sp.94, *Lactobacillus* Sp. 98 out of which 90^{th} sample showed the best results. Further after ammonium sulphate precipitation of the CFS also showed a considerable zone of inhibition of 10mm for the 90^{th} sample at 0-40% concentration..The study indicates the role of food grade lactobacilli gainst C.ssakazakii which can be used in any food matrix to inhibi this pathogen however further studies are needed to eluicidate the active component in cell free supernatant of LAB.

CHAPTER 6

REFERENCES

REFERENCES

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