EVALUATION OF COMMERCIAL PROBIOTICS AGAINST *Cronobacter Sakazakii*

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In partial fulfillment of the requirements for the award of degree of

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By

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CERTIFICATE OF ORIGINALITY

This is to certify that the work submitted in this thesis entitled "**EVALUATION OF COMMERCIAL PROBIOTICS AGAINST *Cronobacter sakazakii*** " submitted by Anubhav Jamwal in partial fulfillment of the requirements for the award of degree of Masters of Technology in Biotechnology, of Jaypee University of Information Technology, Solan, has been carried out under the supervision of Dr. Saurabh Bansal. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma**.**

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Abstract

Lactic cultures isolated from commercial probiotics were examined for antibiotic, antimicrobial and biofilm inhibition assays. LAB isolated from commercial probiotics has shown the antimicrobial activity against *C. sakazakii* strain. Culture isolated from yakult has shown maximum activity in both heat killed cell free supernatant and in untreated cell free supernatant. Isolate from Bifilac has shown activity in pH neutralized supernatant also along with heat treated and non treated CFS. LAB isolated from yakult have shown resistance against all the antibiotics, LAB isolates from different probiotics have shown resistance, susceptibility and intermediate results against different antibiotics. Biofilm inhibition activity of LAB against *C. sakazakii* was observed with Cell free supernatant of selected LAB isolates. Minimum inhibitory activity was measured and the minimum concentration which is responsible to inhibit the biofilm formation was found to be 50 µl. In co incubation assay the maximum biofilm inhibition was shown by untreated CFS and heat treated CFS where as minimum biofilm inhibition was shown by heat treated CFS. In post incubation assay untreated CFS have shown the maximum inhibition and then heat treated CFS. Minimum inhibition was shown by pH neutralized CFS. Biofilm inhibition was maximum during 12 hr of incubation. with increase in time the biofilm inhibition decreased. i.e. less inhibition was seen during 24 and 48 hr of incubation as compare to the 12^{th} hr of inhibition which was maximum.

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CHAPTER 1

INTRODUCTION

Probiotics are live microorganisms when consumed in certain number, provide health benefits beyond inherent basic nutrition. [1] These when administered in adequate amount , confer a health benefit for host. There are many commercial probiotics available in market and are beneficial as these promote a healthy micro biome, balance immune function, enhance nutrient absorption by enzymes, improve your mood, maintain a healthy body weight and much more.[2] Aging, stress, environmental toxins, and medications (antibiotics) can have indiscriminate effects on our healthy micro flora balance. Nowadays modern diets such as junk food and sugar enriched diet, taking antibiotics can negatively affect balance of bacteria in body. Hence certain species of bacteria increase body's levels of healthy bacteria. As many of immune cells are inhabit in intestinal tract, a healthy and balanced immune response is largely dependent on strong communities of beneficial bacteria existing within the gut environment.[4]

1.1 Major Probiotic cultures-

Bacillus- *Bacillus* is a genus of Gram-positive, rod-shaped bacteria that is found widespread in the environment. As a result, they are often called "soil bacteria".Endospores are formed by bacteria under stressful conditions. Dormant bacteria inside is protected by endospores which have tough outer coating. This can be resistant to extreme heat, radiation, extreme freezing, drying, and chemical disinfectants and survive for many years. The endospores converts to a vegetative cell which can thrive when the conditions are favourable for growth.

Lactobacillus acidophilus- *Lactobacillus acidophilus* means rod-shaped, lactic-acid producing bacteria which require an acidic environment. The lactose-digesting capabilities of *L. acidophilus* depend on the numbers of live bacteria and the conditions they live in, *Lactobacillus acidophilus* can utilize, at least in small amounts, many different sugars, from easily digested ones to sugars that humans cannot digest, can produce low ph and bacteriocins hence rendering the growth of pathogenic microorganisms.

Bifidobacterium- *Bifidobacterium* is a genus of Gram-positive, anaerobic, non-sporulating, usually branched rod-shaped bacteria. These bacteria are present in the gastro-intestinal (GI)

tract of newborns since birth and dominate in the intestines in breast-fed babies. This is one reason why the status of probiotics in the mother in pregnancy is very important. Producing both lactic and acetic acids and other beneficial compounds makes *bifidobacteria* an important Probiotic candidate.

Streptococcus- *Streptococcus* is the genus of Gram-positive, non-sporulating, spherical-shaped, chain-forming, lactic-acid bacteria. They are facultative or obligate anaerobes. They ferment glucose to lactic acid. These live on skin and on mucous membranes inside your body, but they can translocate to inner tissues. Some species of *Streptococcus*, such as *S. pyogenes* and *S. pneumonia*, can be particularly pathogenic because of the toxins they produce.

Saccharomyces- *Saccharomyces* means "sugar fungus", and many of the species are used in the food industry. Among non-toxic and non-pathogenic strains of *S. cerevisiae* ,many ethnic fermented dishes and drinks around the world are made using these strains apart from using these in baker yeast.

1.2 *Cronobacter sakazakii*- The pathogen

Cronobacter sakazakii is an opportunistic pathogen which is responsible for serious diseases in adults as well as infants with more severe invasive infections in neonates such as meningitis and necrotizing enterocolitis. *Cronobacter sakazakii* is gram-negative facultative anaerobe, motile, rod-shaped, non-spore-forming pathogenic bacterium which was earlier known as Enterobacter sakazakii. There are eleven species of genus *Cronobacter* among which *C. condiment* is the only speicies which is not reported to cause any disease condition. *Cronobacter* eleven species include *C. sakazakii, C. malonaticus, C. dublinensis, C. turicensis, C. muytjensii, C. condimenti, C. universalis, C. helveticus, C. zurichenesis, C. pulveris and C. colletis*.[3] *Cronobacter sakazakii* have been associated with disease outbreaks and sporadic infections, particularly in premature and immunocompromised infants. Infant formula milk is the only food source that has been epidemiologically linked to disease outbreaks caused by *Cronobacter sakazakii*. Currently there is a dearth of information available on the virulence factors of *C.sakazakii* and the pathogenic mechanisms involved in its neonatal infections. The pathogen possesses an array of virulence factors which aid in tissue adhesion, invasion and host cell injury. Species of *Cronobacter* have been described as ubiquitous bacteria These pathogens have been isolated

from a range of environmental, clinical, food and beverage sources including water, vegetables, cheese and meat Additionally, *Cronobacter* has been isolated from ready-to-eat foods. The presence of *Cronobacter* in these products and subsequent contamination of households increase the potential risks for infections in immunocompromised adults.[4] The risk of *Cronobacter* infection to neonates and immunocompromised individuals is very high, especially in countries with a large immunocompromised (e.g., HIV/AIDS) population. General characteristics of *Cronobacter* including temperature, osmotic and desiccation tolerances as well as possible virulence factors.

C. sakazakki can survive under stressfull conditions by biofilm formation. The pathogen has been reported to form biofilms on enteral feeding tubes, silicon, stainless steel, polycarbonate, glass, and polyvinyl chloride (PVC) in order to survive the stressful growth conditions. There are various factor which supports the formation of biofilm such as flagella, outer membrane proteins, extracellular polysaccharide substances (EPS) and environmental conditions which contribute to biofilm formation.[4]

Highlighting the importance of LAB in controlling *C.sakazakii,* such as *L. acidophilus* or *L. casei* (isolated from faeces of infants) has antibacterial activity against tested *C. sakazakii* strains. They also reported that the inhibition was due to the production of bacteriocins by *L. acidophilus* and *L. casei*. Therefore, this study has been done to evaluate commercial probiotic cultures for their antimicrobial activity against *C.sakazakii* strains.[5]

CHAPTER 2

LITERATURE REVIEW

In this section the relevant representative studies on probiotics and *Cronobacter sakazakii* that appeared in literature during the recent past have been summarized

A.S.Naidu *et al* [2] described that the fermentation involving LAB results in accumulation of organic acids, primarily lactic acid which is end product of carbohydrate metabolism, generated from pyruvate by lactic acid dehydrogenase. The accumulation of lactic acid and the concomitant reduction in pH of the milieu results in a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria. Acetic acid has more antimicrobial activity than lactic acid. *Bifidobacteria* do not produce bacteriocins however, they do produce both acetic acid and lactic acid. The production of these acids reduces intestinal pH, which in turn limits the growth of many potential pathogens and putrefactive bacteria. In the presence of oxygen, LAB produce hydrogen peroxide (H2O2) through electron transport via flavin enzymes. In the presence of H2O2, superoxide anions form destructive hydroxy radicals. This process may lead to peroxidation of membrane lipids, and increased membrane permeability. The resulting bactericidal effect of these oxygen metabolites has been attributed to their strong oxidizing effect on the bacterial cell as well as destruction of nucleic acids and cell protein. LAB strains were reported to produce H2O2 under aerobic conditions in a complex glucose based media. The H2O2 accumulates during oxygen utilization by the cultures simultaneously with an increase in specific activity of NADH oxidase, pyruvate oxidase and NADH peroxidase. H2O2 is not a product of NADH oxidase in vitro, but is formed in substantial quantities from oxygen during oxidation of pyruvate.

Toole *et al* [6] Proper knowledge about gut micro biota and their composition helps us to draw probiotics from narrow range of organism but this trend is changing as particularly given the phylogenetic range and relatively unknown characteristics of the organisms for which the research is going on to use as novel therapeutics. There are some organisms which can provide major health benfits but are not used to provide health benefits, these types of bacteria can be used in future as probiotics and are considered as next generation probiotics. Their development is predominately to be more suitable to a pharmaceutical than a food delivery route. There are certain criterias which are followed by food companies, nutritional supplement companies and

dedicated Probiotic production companies to use the organism in particular product. The Probiotic organisms that are featured in these products have been mainly sourced from the gut or from traditional fermented foods, such as pickles, yoghurts and kefir grains. Generally Regarded as Safe (GRAS) status in the United States has been provided to the more commonly exploited strains/species among the *lactobacilli* and *bifidobacteria*. *Saccharomyces, Bacillus spp., Escherichia coli, enterococci and Weissella spp*. are the other probiotics currently available in the marketplace. Parallel sequencing has enhanced the the knowledge of the composition and function of the human gut microbiome. Although these potential new genera organisms are still at the very early stage of mechanistic investigation but can dramatically extended the range of organisms with potential health benefits. Challenge in future is to use those microorganisms that have not been used as agents to promote health to date, and which are more likely to be delivered under a drug regulatory framework. As with current probiotics, one strategy involves associating the presence or absence of a specific strain with a health phenotype and exploring whether the chosen strain, when administered in sufficient quantities, can recapitulate the health phenotype. The second strategy is to adopt a well-characterized probiotic strain and use them as delivery vehicles for a specific molecule, again choosing the molecule to be delivered based on either a strong association or some mechanistic insight that shows that addition of the molecule would abrogate the disease phenotype and thus promote health. *Akkermansia* , *Bacteroides* and *Faecalibacterium* are the bacteria which have no history of use and are promising candidate for probiotics. There are Production challenges and scale-up challenges which need to be overcome. The *Bifidobacterium* and *Lactobacillus* species that form the mainstay of the commercial supply are anaerobic or microaerophilic organisms, but are much less sensitive to atmospheric oxygen than the strict anaerobes such as *F. prausnitzii*, *Akkermansia muciniphila* and others that are currently being explored as NGPs.

Hajela *et al*.[7] describes that in todays world there is increase in incidences of lifestyle disorders and non-communicable diseases in India. The Indian probiotic market is focusing on preventive health care, diversity in demographics and emergence of well protected environment. Key challenges for probiotics based industries include lack of standardization, product stability and the need for validating product claims. examining the legal and regulatory issues is the need of an hour that would allow probiotic use in India. To improve the method probiotics are marketed in a country and to ensure that probiotic products are made available in an appropriate manner to

the general public, clarity in regulation of probiotic products should be done. In India to manipulate the gut microbiota and advancement in health sector by use of different probiotics remains an exciting proposition. To take this forward several issues are needed to be addressed. These include an improved characterization of the variability in the gut microbiota, a better understanding of how such variability can result in similar or different functional profiles, and more integrative studies that take into account the interaction between the microbiota, the host and the environment to produce a phenotype. Many of probiotics which are claimed to provide gut and immune health benefits are hampered by a relative lack of relevant and validated biomarkers.

Martinez *et al* [8] Probiotics are the microorganisms, which when taken provide health benefit to host. Many recent improvements are done to enhance more probiotics food items and increase their beneficial effect which not only which increase its demand in food industry but also for targeting industrial and pharma . To serve this purpose, development of easy to use, stable and cheap Probiotic microcapsules can become an important key for industrial spreading of microcapsules. By the use of microencapsulation survivability of probiotics under the extreme conditions of gut can be increased which will provide better results in combating the pathogenic microorganisms and will also serves purpose for making certain medicines in health area, Also the monitoring of cell stability along the entire food production including a real storage period as well as the assessment of encapsulated Probiotic metabolism are some topics that require additional investigations. There are some intrinsic factors such as the type of culture selected, growth stage, subcellular injuries by heat or osmotic stress which can affect the probiotics and can vary their variability depending upon the species. The extrinsic factors also plays the role in determining the effectiveness of probiotics such as composition of food matrices, pH value, oxygen level, food manufacturing conditions and storage time. Therefore, also the loss in probiotic viability during gastrointestinal transit, where the principal stressors are the shifting pH and bile, is to be considered as a hurdle that probiotics have to overcome to fulfil their biological role. Hence microencapsulation plays an important role in increasing the survivability and serves as a possible tool to overcome technological hurdles. Selection of capsule materials as well as the technologies adopted in the fabrication of probiotic microcapsules is of great importance because it strictly reflects the final morphological and functional properties of the capsules which determines the effectiveness of probiotics.

Iaconelli *et al* [9] There are some principal factors in microencapsulation which are need to be protected ,these include : Processing conditions (temperature, oxidation, shear, etc.) , Desiccation (for dry food products) Storage conditions (packaging and environment: moisture, oxygen, temperature, etc.). Degradation in the gastrointestinal tract (low pH in stomach and bile salts in the small intestine). Encapsulation process involves the packaging of bioactive compounds like probiotics in this case in mili-, micro- or nano-scaled particles which isolate them and under specific conditions these will be released into environment under control conditions. The sealed capsules coating or shell needs to be semi permeable, strong and also thin which should support the environmental conditions maintaining cells alive, but it can be designed and modified to target or release the probiotics cells in particular area of human body which can be influential in increasing the growth of good bacteria in certain areas or to provide immunity at particular part of our body. In this way, the protection of the biological integrity of probiotic bacteria is achieved during gastro-duodenal transit, achieving a high concentration of viable cells to the jejunum and the ileum.

Sharma *et al* [10] Study was done to assess antibiotic resistance pattern among commercially available Probiotics. *Lactobacillus* rhamnosus, *Lactobacillus* acidophilus, *Lactobacillus* casei, *Lactobacillus* reuteri, *Lactobacillus* plantarum and *Lactobacillus* fermentum , these were the isolates which were studied after isolation. Antibiotic susceptibility tests of these isolates were further performed using disc diffusion method against 45 antibiotics. This study helps us to evaluate the antibiotic resistance in probiotics which we consume regularly and hence this poses a threat of transfer of these antibiotic resistance genes to pathogenic bacteria. Against number of antibiotics, all the isolates were found to be resistant except one strain S9b (L *acidophilus*), when compared with the guidelines given by Clinical and Laboratory Standards Institute (2012). There are different mechanisms of resistance, and it depends upon specific bacterial strain that it will prevail which mechanism, it also depends on a wide array of factors viz. nature of the antibiotic, its target site, the bacterial species itself, including inhibition of cell wall synthesis, inhibition of protein synthesis which includes translation, alteration of cell membranes and inhibition of nucleic acid synthesis. (6.1)

Sahadeva *et al* [11] Study was made to compare the viable number of cells isolated from five difference commercially available probiotics drinks and to check their survivability and tolerance

to ph and bile concentrations by simulation of ph and bile concentration gastrointestinal track. Acid toleration test was done by simulating GI conditions and samples were subjected to different pH levels at a designated incubation time. Estimation of acid tolerance was done by comparing the growth of viable cell counts in MRS agar plates after 48 hours. Mainly all the probiotics were meeting minimum initial count requirement as set by WHO/FAO 2006. For all the different commercial Probiotic drinks taken acid tolerance was same and as level of acidity of acidity was increasing it was having negative impact on the viability of the probiotics. For all the brands, the bile tolerance show the same trend and more inhibitory effect was shown for increasing levels of bile.

Breeuwer *et al* [12] *Enterobacter sakazakii* can be isolated from powdered infant formulas at low level can adapt to osmotic and dry stress. Water activity of dried infant formulas is ca 0.2. Survival of bacteria in such dry environment will depend on the dry stress resistance and osmosis. Intracellular accumulation of ions, like K+, also accumulation of compatible solutes such as proline, glycine, betaine and trehalose to increasing osmolarity is done by bacteria to protect themselves. Trehalose, a polyhydroxyl compounds is able to replace the shell of water covering macromolecules, hence preventing cell damage. As compared to other strains of the Enterobacteriaceae group such as Salmonella and E. coli, Enterobacter sakazakii is relatively resistant to dry stress and osmosis which is further enhanced by addition of compatible solutes. Trehalose, a nonreducing disaccharide of glucose, is assumed to play a pivotal role in protection of bacteria against drying by stabilizing phospholipid membranes and proteins. Nevertheless, in Ent. *sakazakii* the stationary phase is apparently a prerequisite for accumulation of trehalose upon dry stress. The simplest explanation for these observations is that the cells do indeed synthesize more trehalose in the stationary phase, but because of induction of the periplasmatic trehalase in the stationary phase any excess, nonfunctional trehalose is transported to the periplasm, converted to glucose and reused. The existence of such a futile cycle was demonstrated in E. coli K-12, where mutant strains defective in the periplasmatic trehalase accumulated large amounts of trehalose in the medium. The results from this study strongly suggest that trehalose is indeed involved in the resistance to drying of Ent. sakazakii [4]

Khalesi *et al* [13] Probiotics are used for treating many diseases case such as Treatment of intestinal infections, Prevention or treatment of respiratory tract infection, Prevention of CVD

Prevention of osteoporosis, Prevention and/or treatment of female urogenital health Cavities , periodontal disease and halitosis. Hence future investigations aims to develop the methods to provide healthier microbiota, which might help in our physiology and disease processes, will be increasingly stimulated. The probiotic strains should be selected and administered based upon the vitro and in vivo assays done on the micro-organisms of interest, when tested alone or incorporated into a food matrix or a pharmaceutical preparation. Thus, the probiotic strains used for production and the industrial large-scale processing should be adequately characterised and appropriate for each type of product in which they will be delivered, including high viability throughout the storage period and scientific-based evidence for specific health claims. For consumption of probiotics Systematic reviews and meta-analyses are crucial means to assess the strength of scientific evidence for health effects in future and to relate the history of the species used in probiotics to the gut micro biota. The increase in scientific knowledge related to the effects of probiotics on consumer health tends to result in an extraordinary increase in the range of options of this class of functional foods.

Mudroa *et al* [14] Cultured carp demand is growing since last decade due to increase in market growth. For requirement of carp intensive aquaculture system is being practiced and have emerged as promising tool for requirement of carp. During aquaculture animals are subjected to high level of stress conditions which weakens their immune system , hence making them susceptible to diseases conditions. To cope with this situation probiotics are being included in diets as dietary supplement which increases immune response and protect them against various chemical products such as vaccines , antibiotics etc. Immune response of fish is modulated by various intrinsic and extrinsic factor and also from environmental factors among which use of immunostimulants improves the yield of aquaculture by reducing the impact of diseases and stress. When probiotics such as *aeromonas veronii , vibrio lentus* and *flavobacterium sasangense* were included in feed lysozyme C TNF serum protein albumin superoxide anion production and nitric acid production were enhanced in several carp species As these bacteria increases the concentration of good bacteria in gut and sysnthsis of inhibhitory compounds and competeting with pathogenic bacteria renders improved immunity. B.circulans PB7 probiotic when tested to increases the innate immunity in c. catla challenging the fish pathogen A. hydrophilla, b.ciculans increases the phagocytic activity. *Aeromonas veronii , vibrio lentus* and *flavobacterium sasangense* strains were provided to carp for 28 days and results were seen as

enhaced immunity against pathogenic bacteria. Overall results of probiotics feed results in increasing appetite which in turn results in better growth of carp and increased feed utilization.

Mondel *et al* [15] In intestinal barrier function important role is played by Inducible epithelial human-defensins (hBD). Probiotics which have potential to complete with pathogenic bacteria and are considered clinically effective induce antimicrobial hBD-2. In this study author addresses the probiotics survival affect due to defensins in healthy organism in in-vivo study. Various test including pathogenic bacteria were done and assays results showed that all tested bacteria were similarly killed by defensins allowing to analyse the suicidal character of this effect. In Probiotic treatment, defensin induction proved to be an important mechanism for providing immunity against various pathogenic organisms.

Osaili *et al* [16] For the production of probiotics the development of a suitable technology is a major challenge for industrial production, which is an important factor and should be take into account for maintaining the viability and the stability of the organisms involved. Basis for the production of probiotics includes microbial criteria, stress tolerance during processing and storage of the product. Due to changing trends in population demography, consumer affluence, increased education, life expectancy and improved health care give rise to rapidly emerging diet and health conscious clientele production of functional foods is being recognized as the number one food biotechnology industry. Criteria which is need to be fulfilled For organisms to be considered as probiotics includes - It should be isolated from the same species as its intended host, It should have a demonstrable beneficial effect on the host ,It should be non-pathogenic,It should be able to survive the transit through the gastrointestinal tract, On storage, large number of viable bacteria must be able to survive prolonged periods. These are the required properties for probiotics to be effective in nutritional and therapeutic. For probiotics to be helpful therapeutically and nutritionally the selection of organisms would be based on specific properties that are desired. This can be achieved by either classical biological selection techniques or genetic engineering and these approaches are promising to substitute present probiotics with next generation probiotics .Strain must be genetically stable for the maintenance of its favourable properties. For the production of probiotics it is important that the microorganisms multiply rapidly and densely on relatively cheap nutrients and that they remain viable during processing and storage. Viability of probiotics will depend upon the following conditions- their ability to

survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5, hence Tolerance of acid and bile is important parameter. The bactericidal and bacteriostatic activity of the growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested in well-plates against a wide variety of pathogens. Ability to eliminate competitors will enhance ability of probiotics to establish in the gastrointestinal tract Body surfaces are penetrated by pathogenic microorganisms due to a massive attack of the pathogens or to a (temporarily) reduced colonisation resistance hence resistance of colonization is also an important parameter. One of the great challenges is incorporation of the probiotic bacteria into other products apart from the dairy products which includes Probiotic juice. The recognition of dose delivery systems for the Probiotic bacteria has also resulted in research efforts aimed at developing Probiotic foods outside the dairy sector.

Collado *et al* [18] Highlighting the importance of LAB in controlling *C.sakazakii*, looked into the possible relevance of probiotic strains to diminish the risk of a *Cronobacter* infection. They specifically targeted the interaction between *Cronobacter* spp. and human intestinal mucus. However, data demonstrated that the degree of adhesion of probiotic strains to intestinal mucus was not directly proportional to the degree of *Cronobacter* displacement. He also observed that pH-neutralized cell-free supernatant (CFS) of *L. acidophilus* or *L. casei* (isolated from faeces of infants) has antibacterial activity against tested *C. sakazakii* strains. They also reported that the inhibition was due to the production of bacteriocins by *L. acidophilus* and *L. casei*. Recently, Sharma and Prakash 2013 demonstrated 50% reduction in *C. sakazakii* colony counts by *L. fermentum, L.casei* and *Pedicoccus acidilactici* at five minutes of contact time.

Zhong *et al* [19] have reported the suppressive effects of L.rhamnosus GG on the adhesion and invasion of *Cronobacter* in Caco-2 cells, where LRGG produced a concentration-dependent inhibition of *Cronobacter* adhesion and invasion by competing with and excluding the latter for cell adhesion. The preventive effect was further investigated in neonatal rats where LRGG inhibited entry across the intestinal barrier preventing meningitis in neonatal rat model.

Charchoghlyan *et al* [20] Recently, *L.acidophilus* n.v. Er2 317/402 strain Narine"has been reported for its antimicrobial activity against *C.sakazakii* due to greater proportion of acid molecules in undissociated form in the supernatant of lactic culture, which rapidly enter *Cronobacter* cells and increase levels of cytoplasmic acidification in bacteria . However,

the effectiveness of probiotic LAB is considered to be population-specific due to variation in gut microflora, food habits and specific host-microbial interactions. The limited studies on anti-*Cronobacter* activity of probiotics emphasize the screening of potential LAB in particular bifidobacteria for their anti-*Cronobacter* potential and the mechanism involved.

Hancock *et al* [21] describes about the biofilm forming potential of *E.coli* strain *Nissle* 1917. *Nissle* 1917 can be used as probiotic as it provides health benefits to host and helps in treatment of a wide range of gastrointestinal infections. This strain biofilm formation capability was good than other enteropathogenic, enterotoxigenic and enterohaemorrhagic E. coli strains, Also it was able to outcompete most pathogenic strains during biofilm formation. Completing for nutrition, adhesion against pathogenic strains makes *Nissle* 1917 a beneficial bacteria hence its number in gut should enhance for the protection against pathogenic organism.

Patrone *et al* [22] describes that "Effectiveness of probiotics are strain specific and inhibitory effect of probiotics mainly depends upon their synergetic effect on pathogenic bacteria." The efficacy of probiotics depends upon strain-specific features and the number of viable cells, where several reports of deviations from the label in the actual content of strains in probiotics products are a matter of concern. Different methods were used for the identification of species present in probiotics products but results shows deviation for actual number mentioned on probiotic product . Identification of species was done using PCR amplification and sequence analysis of the 16S rRNA gene, and determination of the total amount of species present in the products was done using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis followed by DNA sequencing of the excised bands. Plate count methods demonstrated poor correlations between quantitative label indications and bacteria recovered from plates . Outcomes of this study strongly suggest that of the five commercially available probiotic supplements from India and Pakistan claiming to contain *Bacillus* clausii spores, only Enterogermina complies with label indications. Periodic surveillance of label claims of approved food and therapeutic probiotics is essential to ensure the safety and efficacy of for life products.

Singh *et al* [23] Molecular characterization, and antibiotic susceptibility of *Cronobacter* spp. was determined in following study exploring Dairy or non-dairy based products. Different food samples, infant food formula and different plants and herb were tested for presence of

Cronobacter spp. The *Cronobacter* spp. prevalence was highest in herbs and spices (34 %)while environmental samples had contamination rates of 23 % indicating plants as a possible reservoir of this pathogen.

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CHAPTER 3

Material and methods

3.1 Isolation of cultures from probiotics products

Six different commercial probiotic supplements marketed in India were collected from local retailers (Table-3.2). These probiotics were containing blend of aerobic and anaerobic bacteria. Vibact and Bifilac were in powered formulation (sachets), Vizylac, rinifol and Caplac were in tablet formulation and yakult was in liquid formulation. The LAB from these products were isolated on de Man Rogosa Sharpe broth (MRS) agar plates.[24] The individual colonies obtained on MRS agar were further subcultured on MRS agar to get pure cultures. The cultures were tentatively identified based on Grams reaction and catalase test. Colonies were differentiated according to their shape, size and other morphological features.

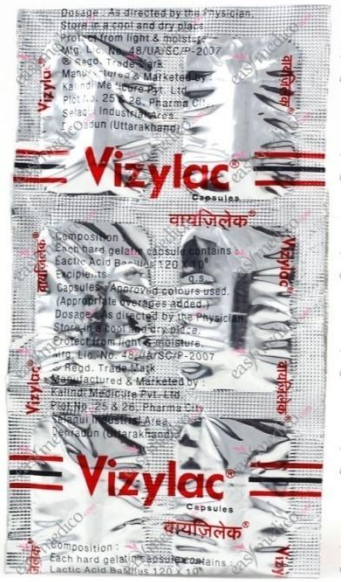
PROBIOTIC PRODUCTS	**COMPOSITIONS**
BIFILAC	*Lactobacillus sporogenes* : 50M cells, Streptococcus faecalis : 30M cells, Clostridium butyricum : 2M cells, *Bacillus* mesentericus : 1 million cells
VIZYLAC	*Lactobacillus* Sporegens - 40 Million Spores
VIBACT	*Bacillus mesentericus* -1M spores, *Clostridium butyricum-2*M spores, *and Lactobacillus sporegens*-50M spores , Strep Faeecalis- 30M spores.
RINIFOL	*Lactobacillus*- 40M spores.
CAPLAC	*Lactobacillus sporegens- 60*M spores.
YAKULT	*Lactobacillus casei Shirota*

Table 3.2

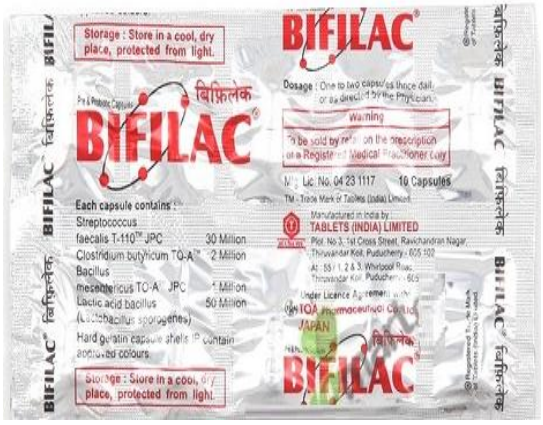
Fig- 3.3 Commercially available Probiotic products

YAKULT

VIBACT



VIZILAC



BIFILAC

The individual colonies obtained on MRS agar were further subcultured on MRS agar to get pure cultures. After pure colonies were obtained glycerol stocks were made and were stored at -20°C.

The *Cronobacter sakazakii* isolates used in this study were already reported from our laboratory [29]. All the strains, available as glycerol stock were revived of *Cronobacter sakazakii*, were sub-cultured in Trypton soy broth and checked for purity. The standard stain of *Cronobacter* was further tested against different strains of isolated probiotics.

Determination of colony forming unit (CFU)

Lactic cultures were inoculated in MRS broth for overnight incubation. After giving overnight incubation different serial dilutions were made in test tubes. 4^{th}, 5^{th} and 6^{th} serial dilution was selected and plating was done to the number of colonies. Optical density (OD) was taken of selected dilution and CFU was determined.[24]

Similar procedure was followed for *Cronobacter sakazakii* (E604) to calculate the colony forming unit (CFU). *C. sakazakii* was given the incubation of 3 hrs, followed by the selection of dilution factor of culture for plating on Enterobacter sakazakii medium and optical density (OD) was measured. Hence after 3hrs at particular OD, colony forming unit of bacteria was determined.

3.4 Antibiotic susceptibility test for isolated probiotics cultures

Resistance to commonly used antibiotics was assessed by disk diffusion method. These resistance attributes are often non transmissible and intrinsic however some lab may carry potential transmissible plasmid encoded antibiotic resistance gene.[25] Disk diffusion method was used to determine the susceptibility of different isolates to antibiotics as per the recommendations of National Committee for Clinical Laboratory Standards (NCCLS, 2008).[27] The overnight grown cultures of probiotics isolates (OD = 0.5) were spread on Muller Hinton agar (Hi media Laboratories, Mumbai, India) plates. The antibiotic discs were placed on the cultured plates and incubated for 24 h at 37°*C*. The results were expressed as sensitive (S) and resistant (R) by measuring the diameter of inhibition zones as per EFSA, 2008.

3.5 Antimicrobial activity of probiotics against *Cronobacter sakazakii*

During fermentation process, types and levels of organic acid produced depends upon strains, culture composition and growth conditions [28]. Antimicrobial compound like organic acids, H2O2, CO2, diacetyl and bacteriocins can play role in inhibition of activity of pathogen. H2o2 can cause denaturartion of enzymes due to oxidation of sulfhydryl groups and from peroxidation of membrane lipids thus increasing membrane permeability. [28] Bacteriocins exert their lethal activity through adsorption to specific receptors which are located on the external surface of sensitive bacteria, renders metabolic, biological and morphological changes resulting in the killing of such bacteria.[2] The antimicrobial activity of cell free supernatant of overnight grown probiotic cultures was tested against *Cronobacter sakazakii* strains using agar well assay. The overnight grown probiotic strains in MRS broth were centrifuged (2000×g for 5 min) and the spent medium was collected in fresh tubes. The spent culture medium was filtered through 0.22 μM cellulose membrane filters (Millipore, USA). The CFS obtained was given two treatments: Neutralization to a pH of 7.0 by addition of 5 N sodium hydroxide (NaOH) solution and a heat treatment of 100°C for 5 min to obtain the heat-treated (H-CFS) preparation. All the three CFSs (CFS, N-CFS and H-CFS) were stored at -80^{o}C until further use. The un-inoculated MRS broth and its heat-treated preparation (H-MRS) were taken as control.

For antimicrobial assay, the indicator strains of *Cronobacter sakazakii* were grown to exponential phase (4 h to an OD= 0.5) in Tryptone soy broth (TSB) and spread on the TSA plates. After spreading, wells were punched in the agar plates and the wells were filled with 100 ul of all the three CFS preparations alongwith the control MRS broth. The plates were incubated at 35 ºC for 12 h and the diameter of zones of inhibition (mm) was determined.

3.6 Minimum inhibitory concentration of cell free supernatant

Antimicrobial drug which is able to inhibit the growth of pathogenic organism at its minimum concentration is know as its minimum inhibitory concentrations. This minimum inhibitory concentration can be further use to make different drug formulations. MIC was determined in

microtitre plate assay.[28] The minimum inhibitory concentrations (MICs) of CFS were determined in microtitre plates by micro dilution assay as per the protocol of Clinical and Laboratory Standards Institute (CLSI). The untreated CFS was diluted in the range of 0.13-0.250 in TSB. Briefly, a bacterial inoculum (10 µL, OD=0.5) was added the microtitre plates, followed by addition of different concentrations of CFS. TSB was used as a negative control. The final volume of each well was 250 µL. The plates were incubated at 37°C for 24 h and absorbance was measured at 600 nm. The MIC was defined as "the lowest concentration of CFS which can inhibit visible growth of microorganism".[29]

3.7 Anti-biofilm activity of the CFS

The anti-biofilm potential of CFS from isolated probiotic strains was determined in polystyrene, flat-bottom 96-well microtiter plates (MTP) using crystal violet (CV) staining assay (Singh et al., 2016).[30] In brief, a 10 µl of cell suspension of indicator strain of *C.sakazakii* (OD600 nm =0.5) incubated with CFS, N-CFS and H-CFS preparations (30 µl each) in two different strategies as inhibition of biofilm formation (co-incubation) and disruption of mature biofilms (post incubation). For co-incubation assay, the CFS from different stains and indicator strain were added simultaneously to MTP and co-incubated for 24 h at 37^{o}C. For the ability of CFS to disrupt mature biofilms, the test strains were added to MTP and allowed for form biofilm for 48 h. Post incubation, the CFS was added to the wells and incubated further for 24 h. After incubation, the planktonic cell suspension was aspirated and wells were washed with phosphate buffered saline (1X PBS) to remove loosely adherent cells. The surface adhered cells were stained with 200 µl of 0.2% CV solution for 15 min at room temperature (D.Djordjevic et al. 2002). Following staining, CV solution was decanted and the stained cells were solubilized with 250 µl of 33% glacial acetic acid.[31] The biofilm biomass was then quantified by measuring the intensity of CV at OD_{570nm} using a Go skan microplate reader (Thermo, USA). The anti-biofilm activity (%) was calculated following the formula: anti-biofilm activity (%) = (Control $_{OD570\ nm}$ − Test $_{OD570\ nm}$ / Control $_{OD570\ nm}$) × 100. The uninoculated MRS broth was taken as negative control.

3.8 Visualization of biofilms

Light microscopic analysis

The biofilms of indicator *C.sakazakii* strains were developed on glass cover slips. The strain was grown in 6 well tissue culture plate containing sterile glass cover slips, TSB (1680 µL/well) and CFSs from different lactic strains (240 µL/well). The wells without CFS were taken as control. The plates were incubated for 48 h at 37^{o}C. After 24 h, the planktonic cells were removed and cover slips were rinsed with phosphate buffer (pH 6.5) and were stained with 0.2% CV solution for 30 min.[31] The stained cover slips were visualized under light microscope at 40X magnification (Olympus Microscope, USA).

Fluorescent Microscopy

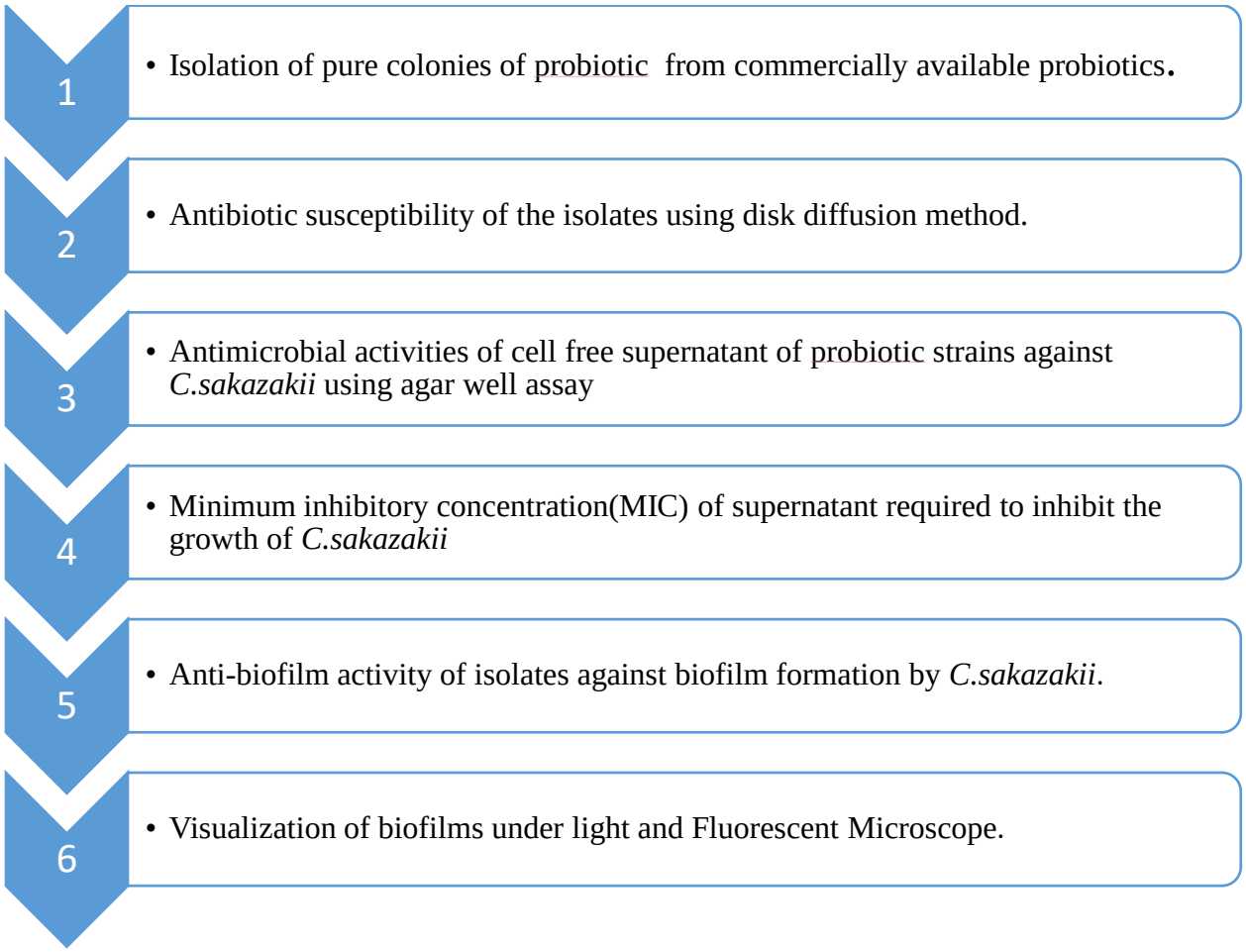
For the fluoresecent microscopic images, the biofilms were treated as discussed before. After the treatment of biofilms, the cover slips were stained with LIVE/DEAD *Bac* Light bacterial viability kit (L10316, Invitrogen-Molecular Probes, USA). The kit included mixture of two fluorescence dyes: SYTO 9 (green-fluorescent nucleic acid stain) and propidium iodide (red-fluorescent nucleic acid stain). The viable bacterial cells with a healthy or intact membrane stain green, whereas dead cells or cells with a damaged membrane stain fluorescent red.[31] The stained coverslips were observed at 400X magnification using a fluorescence microscope BX53 (Olympus Microscopy, USA) equipped with imaging system Qiclick^{TM} (Olympus, USA).

Statistical analysis

Each experiment was conducted in triplicate at three different time points and the data were represented as mean values. The statistical significance among the treatments was conducted using ANOVA and Tukey's multiple comparison test (p<0.05) with SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Efficacy of LAB CFSs to inhibit biofilm formation under co-incubation and post-incubation strategies was checked.

METHODOLOGY FLOWCHAT

Fig 3.9



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CHAPTER 4

RESULTS

Single colonies were obtained of from commercially available probiotics were tested against strains of standard strain of *Cronobacter sakazakki* (E604)

Product	**Isolate obtained on MRS agar**	**Date of manufacture**	**Expire date**	**Lot number**
Bifilac (powder**)**	*Lactobacillus species*	**May-2017**	**Oct-2018**	**ALA711**
Vizylac (capsule**)**	*Lactobacillus Sporegens*	**Nov-2017**	**Apr-2019**	**DVC-17096**
Vibact (powder**)**	*Lactobacillus Species*	**Oct-2017**	**March-2019**	**BA7O2**
Rinifol (capsule**)**	*Lactobacillus*	**July-2017**	**Feb-2019**	**CDD110619**
Caplac (capsule)	*Lactobacillus Sporegens*	**Nov-2016**	**April- 2018**	**10916011000167**
Yakult (liquid**)**	*Lactobacillus casei shirota*	**20 May-17**	**30 June-17**	**11110.MFA**

TABLE 4.1

Table showing he manufacture and expiry dates of commercial probiotics along with the lot number. Seven different colonies were selected from these commercially available probiotic products, two different types of colonies were recognized based on morphological features from

yakult and five from rest other probiotic products. Since only aerobic conditions were provided for their growth anerobic species were not targeted for studies.

4.2 Antibiotic susceptibility of lactic cultures

From the experiment results we can conclude that all the strains of yak 2 cultures are resistant against all antibiotics.

All the probiotics isolates are resistance against clindamycin.

- Isolates from Bifilac, vizilac, refinol, caplac and vibact are susceptible to chloramphenicol, tetracycline, ampicillin and resistance towards clindamycin and intermediate towards amikacin and streptamycin.
- Isolates from Bifilac, vizilac, refinol, caplac and vibact are susceptible against cephalothin and vanomycin.
- All isolates are resistance against novobiorin except vibact isolate which is intermediate.
- All isolates are susceptible against chloramphenicol except yakult isolate and refinol isolate.
- Against rifampicin susceptible strains are isolates of yakult, bifilac, vibact and caplac and resistant strains are isolates of vizilac, refinol and yakult2.
- Against amoxyclav susceptible strains are isolates of bifilac, vizilac, vibact and caplac and resistant strains are isolates of yakult and refinol.
- All strains are resistant against bacitracin except isolate of yakult and vibact.
- Against gentamicin all strains are susceptible except isolates of yakult2 and vizilac.
- For cephalothin isolate of bifilac is intermediate , vizilac and yakult2 isolates are resistant rest all isolates are susceptible.
- For amikacin, yakult1 isolate is susceptible, yakult2 isolate is resistant and rest all isolates are intermediate.
- For ampicillin, all isolates are susceptible except isolate from yakult.

Susceptible, Resistant and Intermediate patterns are shown in table 2.2 below.

Isolates	Source	C	RIF	AMC	B	TE	HLG	E	NV	CB	VA	CEP	AK	CD	S	AMP
Lactobacillus species	YAK	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S
Lactobacillus species	YAK2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Lactobacillus species	BIFI	S	S	S	R	S	S	R	R	S	S	I	I	R	R	S
Lactobacillus Sporegens	VIZI	S	R	S	R	S	R	S	R	S	S	R	I	R	I	S
Lactobacillus species	REF	R	R	R	R	S	S	R	R	S	S	S	I	R	I	S
Lactobacillus species	VIB	S	S	S	S	S	S	R	I	S	S	S	I	R	I	S
Lactobacillus sporogens	CAP	S	S	S	R	S	S	R	R	S	S	S	I	R	I	S

Zhou *et al* [25] **TABLE 4.3**

S= Susceptible, **R**= Resistant, **I**=Intermediate

C (chloramphenicol)- 30units/discs RIF(rifampicin)- 5mcg/disc

AMC-(amoxyclav)-30mcg/disk, B (bacitracin)- 10units/disk

TE (tetracyclin)-30mcg/disc, HLG(gentamycin)-120mcg/disc

E(erythromycin)-15umits/disk NV(novobiorin)-30mcg/disk

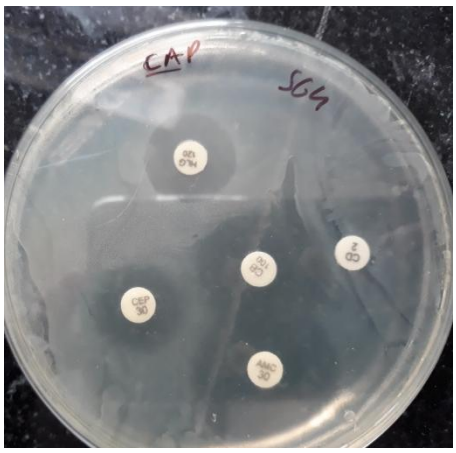
CB(carbenicillin)-100mcg/disk, CEP(cephalothin)-30mcg/disk

Ak(amikacin)-30mcg/disk, CD(clindamycin)-2mcg/disk

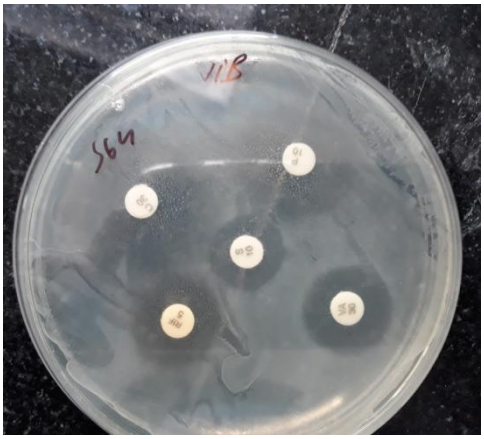
S(streptamycin)-10mcg/disk. AMP(ampicillin)-30mcg/disk

VA(vancomycin)-30mcg/disk.

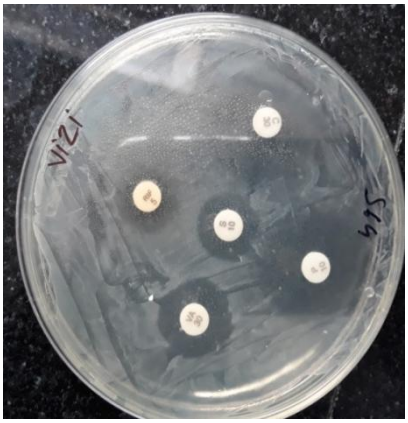
Fig 4.4 Antibiotic susceptibility Images of Lactic cultures



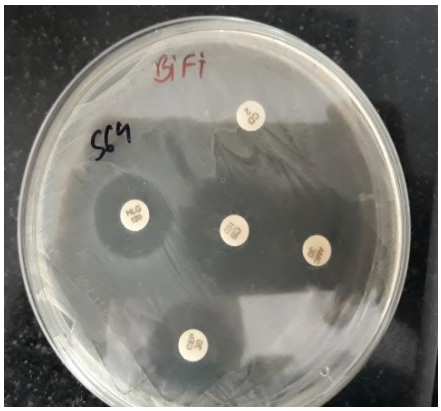
CAPLAC



VIBACT



VIZYLAC



BIFILAC

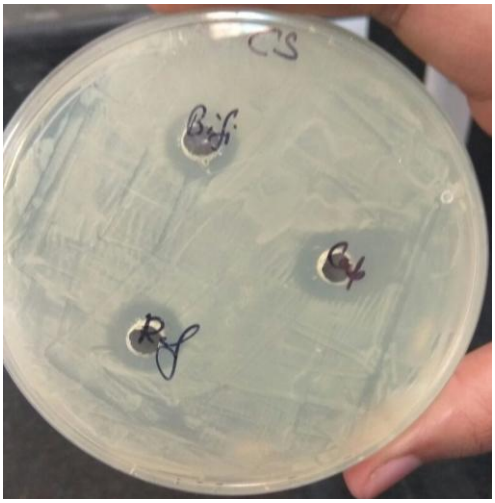
4.5 Determination of key component for antimicrobial activity

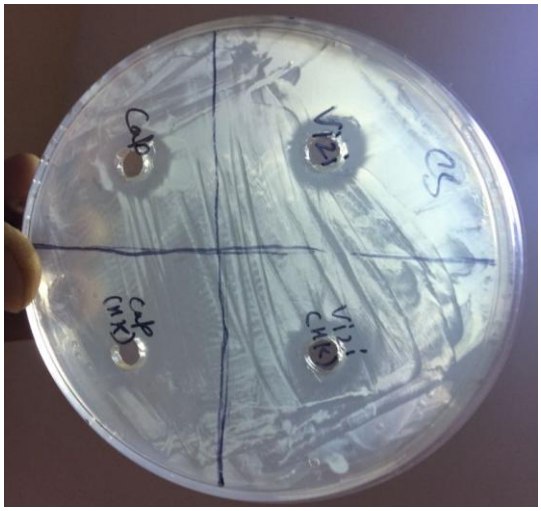
Results obtained from antimicrobial activity shows that all the cultures cell free supernatants were inhibiting the growth of *Cronobacter sakazakii*. Heat neutralized cell free supernatants of all cultures were also inhibiting the growth of *Cronobacter sakazakii*. This shows that lactic acid was involved in inhibition activity for all probiotics culture except isolate from Vizilac sachet. In case of Vizilac Sache isolates, pH neutralized supernatant was also showing inhibition.

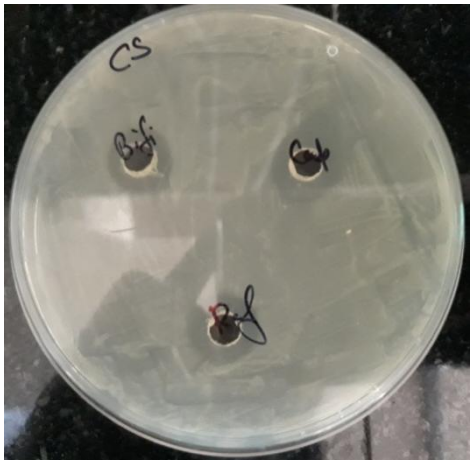
Isolates	Source	Supernatant	Heat neutralized	pH neutralized
Lactobacillus species	Yakult	12.33±0.5	11.33±1.1	-
Lactobacillus species	Yakult 2	15±1	12.66±0.5	-
Lactobacillus Sporegens	Vizylac	12.66±1.1	11.33±1.1	-
Lactobacillus species	Bifilac	12.33±0.5	11.33±0.5	9.66±0.5
Lactobacillus species	Rinifol	10±0	10.33±0.5	-
Lactobacillus species	Vibact	10.66±0.5	10±0	-
Lactobacillus Sporegens	Caplac	14.33±0.5	12.33±0.5	-

TABLE 4.6- Showing the antimicrobial activity of cell free supernants against *Cronobacter sakazakii*

Fig- 4.7 Images of different lactic cultures showing the inhibiton of *Cronobacter sakazakii*







After antibiotic susceptibility and antimicrobial activity of lactic cultures, lactic cultures cultures which were showing best results in inhibtion of *Cronobacter sakazakii* were selected and futher experiment was conducted using isolates from vibact, yakult , bifilac and caplac.

4.8 Minimum inhibitory concentration of assay

MIC was determined by microtitre plate assay.(k.spinler et al 2008).

ISOLATES	SOURCE	CFS (30µl)	CFS (40 µl)	CFS(50 µl)
Positive control	Positive control	2.99±0.1	3.01±0.1	3.01±0.1
Lactobacillus species	Vibact	2.43±0.3	1.61±0.2	0.02±0.04
Lactobacillus species	Yakult	3.05±0.08	2.20±0.1	0.26±0.1
Lactobacillus species	Bifilac	2.53±0.3	1.84±0.2	0.43±0.05
Lactobacillus sporogens	Caplac	2.59±0.3	1.7±0.1	0.21±0.3

From Table 4.8 of MIC assay we can conclude that 50 µl conc. of probiotics isolates are required for inhibiting the activity of *Cronobacter sakazakii*.

4.9 Biofilm inhibitory assay

Biofilm assay was done to inhibit biofilm formation (co incubation) and mature biofilm (post incubation).

Results obtained from co incubation (i.e. when cell free supernatant of lactic cultures is added at same time along pathogen) are shown in table 4.10

ISOLATES	Source	CFS	hCFS	nCFS
Lactobacillus species	Vibact	0.004±.002	0.003±.002	0.004±.001
Lactobacillus casei Shirota	Yakult	0.01±0	0.003±.001	0.006±.003
Lactobacillus species	Bifilac	0.004±0	0.003±0	0.003±.002
Lactobacillus sporogens	Caplac	0.01±.002	0.004±.001	0.004±.001

TABLE 4.10

Inhibition of biofilm can be seen form the data shown in Table 4.10 obtained after co-incubating supernatant and pathogen for 24hrs.

Post-incubation assay-

ISOLATES	**Source**	**12hr**			**24hr**			**48hr**		
		CFS	**hCFS**	**nCFS**	**CFS**	**hCFS**	**nCFS**	**CFS**	**hCFS**	**Ncfs**
Lactobacillus sporogens	Caplac	44±0.3	31±0.1	19±0.5	57±0.4	73±0	56±0.4	57±0.4	73±0.1	59±0.4
Streptococcus faeecalis	Bifilac	85±0	28±0.1	14±0.1	62±0.5	50±0.1	55±0	62±0.5	46±0.2	57±0
Lactobacillus sporogens	Vibact	96±0.1	53±0	44±0.1	48±0.3	50±0.2	64±0.1	51±0.2	58±0.1	66±0.1
Lactobacillus species	Yakult	82±0	42±0.2	23±0.2	57±0.3	37±0.2	46±0.7	56±0.2	44±0.2	49±0.7

. **TABLE 4.11**

Inhibition of biofilm can be seen form the data shown in table 4.11. Percentage inhibition is calculated after the interval of 12hrs, 24hrs, and 48hrs to study the comparative analysis of effectiveness of lactic culture supernatant in inhibiting the mature biofilm.

4.12 Visualization of biofilms results-

Light Microscopic Images-

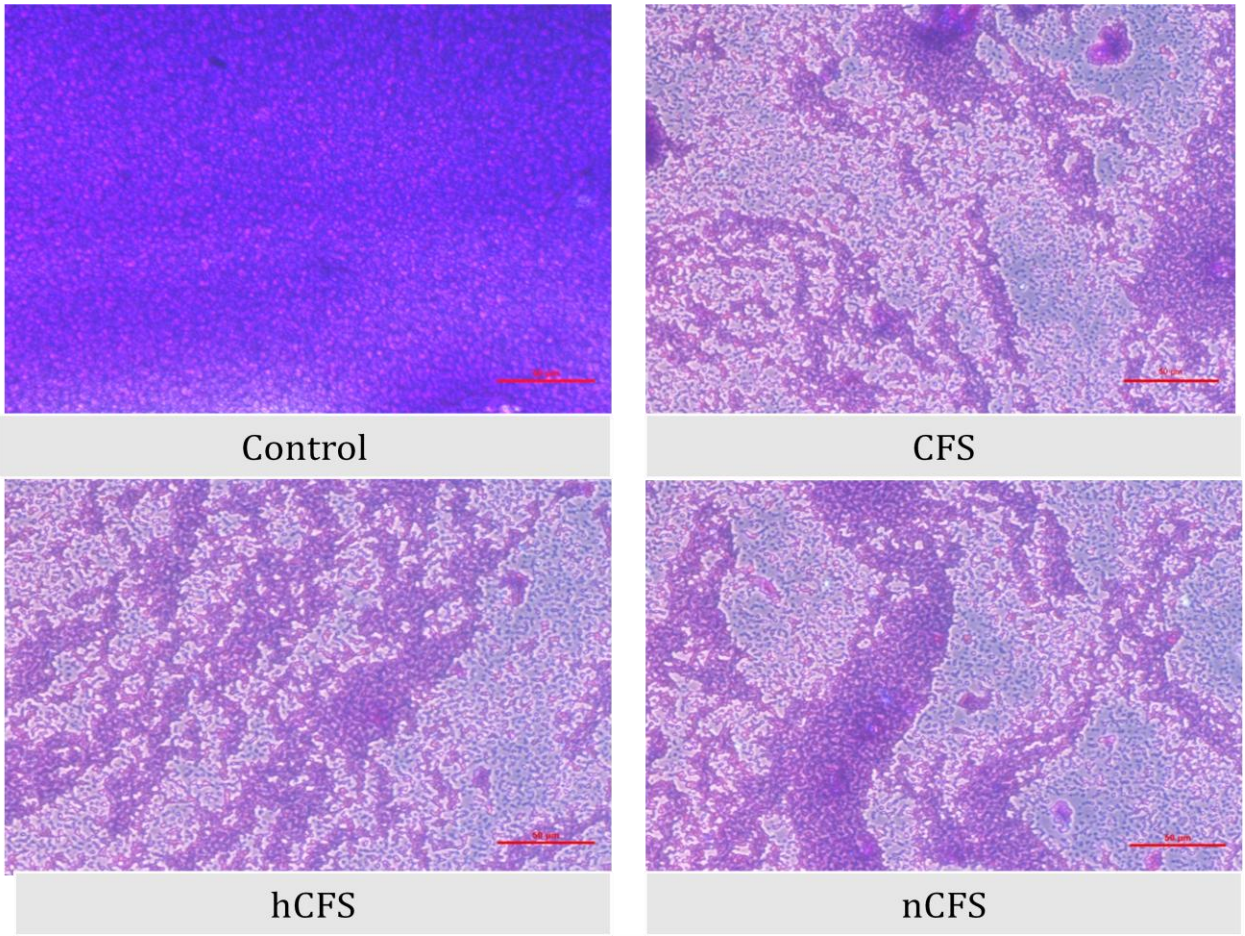


Fig 4.12(a)- Bacterial biofilm stained with crystal violet showing the inhibitory activity of cell free supernatant of probiotics against biofilm formed by *Cronobacter sakazakii*. Inhibition can be clearly seen in biofilm treated with CFS, heat treated CFS (hCFS) and pH neutralized CFS (nCFS) as compared to control.

Fluorescent Microscopy Images-

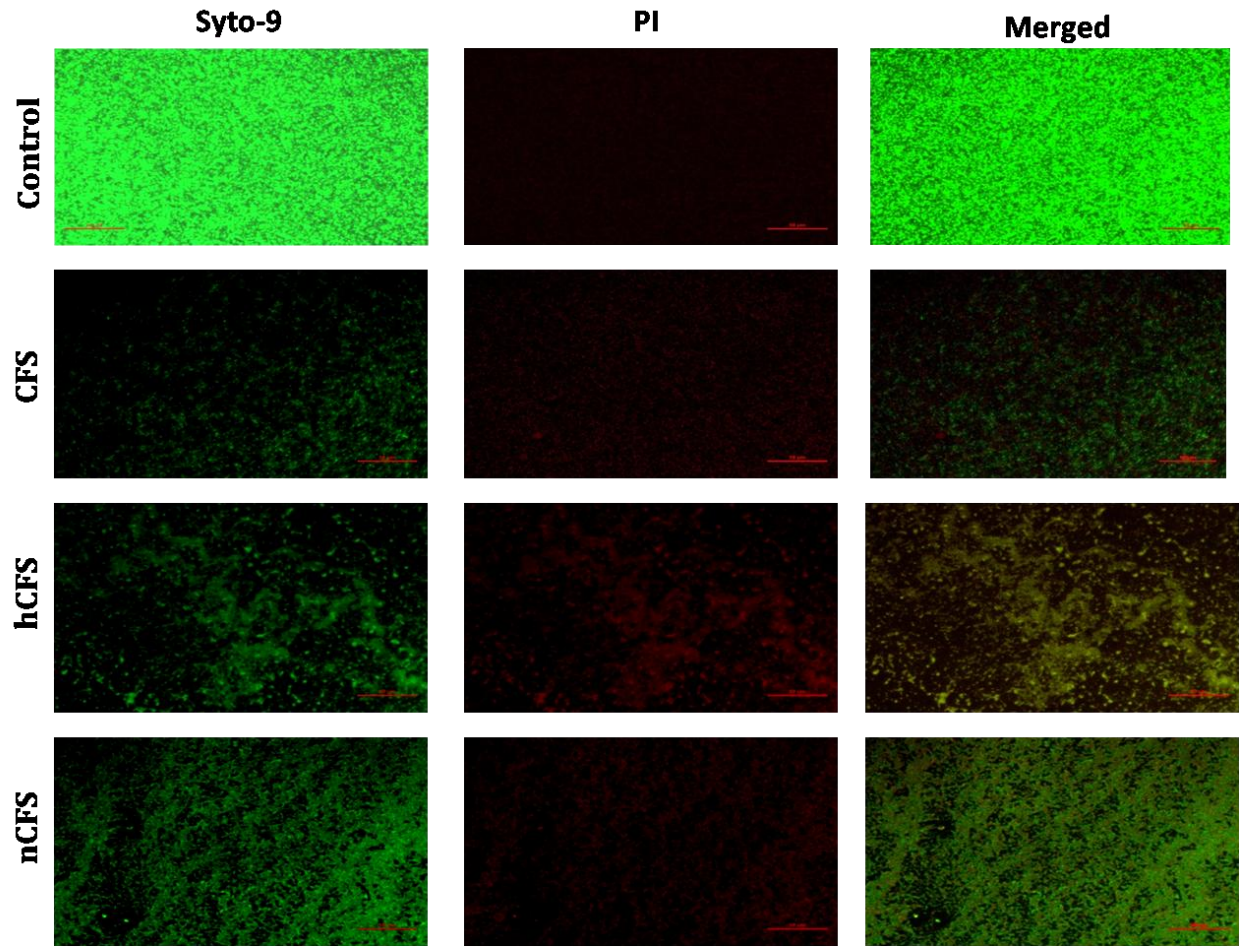


Fig.4.12 (b) Fluorescent microscopic images of biofilms of *C. sakazakii* grown in the absence and presence of treated and non treated cell free supernatant of probiotic bacteria. lane 1 treated with syto 9, lane two visualized with pi stained dye and lane 3 is merged image.

DISCUSSION

Lactic cultures isolated from commercial probiotics are seen to inhibit resistant and susceptible pattern against some most commonly used antibiotics. These probiotics which tend to show resistance pattern may have some genes coding for resistance factor present in the plasmid of bacteria.[29] These genes which are responsible for resistance against antibiotics can be transferred from these probiotics to other pathogenic bacteria hence poses the serious threat. There are many reported cases of isolates of commercial probiotics showing the resistance toward many commonly used antibiotics.[32] Although resistance to antibiotics can be beneficial for these good bacteria to maintain their high population in gut which inturn is beneficial for health, but when plasmid carrying resistance factors are transferred to pathogenic bacteria, it becomes the great challenge to eliminate them. The role of lactic cultures in inhibiting the pathogenic microorganisms and increasing the immunity is related to their ability to competitive adhering to surface, completing for nutrient requirement against pathogens, inhibiting the biofilm formation and release of antimicrobial substances such as bacteriocins, lactic acid and acetic acid, hydrogen-peroxide and carbon-dioxide[31].The supernatants obtained after removing cells was given heat shock and was also Ph neutralized, results showing that mainly lactic acid was involved in the inhibition of *Cronobacter sakazakii* as bacteriocins were destroyed by heat treatment. But in case of vizylac, pH neutralized and heat treated supernatant both inhibited the *Cronobacter sakazakii* hence some other antimicrobial susbstance results in inhibition. The role of probiotics is to provide the immunity against various pathogens and improves the immune system but these are influential only when administered in certain amount for considerable amount of time. Each Probiotic is strain specific and is needed to administered in adequate quantity to inhibit the growth of pathogen. Antibiotics are prescribed to kill the the pathogen at latent stage of infection but intake of probiotics can decrease the risk of occurrence of infection hence will reduce the dependence or intake of antibiotics. Gut micro flora gets enriched by useful bacteria with the help of probiotics which are killed by the use of antibiotics. Pathogenic bacteria tends to form biofilm through quorum sensing.[33] As seen under the microscopic analysis of biofilm formed by *Cronobacter sakazakii* and biofilm inhibition assay, reduction in biofilm formed by the pathogen is observed. Even mature biofilm is inhibited by supernatants of lactic culture shows the effectiveness of probiotics in combating the pathogens and reducing the risk of diseases. Hence, probiotics are not only effective in treating diarrheal cases but can also inhibit

the growth of pathogens causing other diseases such as meningitis which is caused by *Cronobacter sakazakii*. Future of probiotics seems to be more promising in pharmaceutical field along with its application in food industry.[32]

CHAPTER-5

SUMMARY AND CONCLUSION

Commercially available probiotics were collected from the market. Different probiotics products were in the form of powder (sache), liquid and capsules. These probiotics comprised of blend of bacteria of specific strains. From these probiotics, pure colonies of lactic acid bacteria were isolated on MRS Agar. Isolated pure colonies were sub-cultured on MRS broth and maintained as glycerol stocks. The Cronobacter sakazakii strains were procured from our laboratory and maintained in TSB. The isolated probotics were tested for antibiotic susceptibility test was done against commonly used antibiotics. Based on the diameter of zone of inhibition, the isolates were catagoriesed as susceptible or resistance towards particular antibiotic.

For the activity against *C.sakazakii*, the cell free supernatant (CFS) was obtained from pure cultures by centrifugation. This cell free supernatants (CFS) was divided into different parts which includes heat treated supernatant (hcfs), pH neutralized supernatant (ncfs) and untreated supernatant. These supernatants were tested against *Cronobacter sakazakii* standard strain (E604). The antimicrobial activity of lactic cultures was tested and evaluation was made based on diameter of zone of inhibition. Four lactic cultures were selected which showed the best results in inhibiting the *Cronobacter sakazakii*.

Followed by antimicrobial activity, the Minimum inhibitory concentration (MIC) of these selected lactic cultures was determined with different doses of CFS (30-50µl) using microtitre plate assay. A MIC value of 50 µl of CFS was determined against *C.sakazakii* strain.

After MIC determination, a 50 µl of CFS was tested for its anti-biofilm activity using standard crystal violet plate assay. The percentage inhibition of biofilm was calculated and comparison was made to know the effectiveness of different culture in inhibiting the biofilm formed by pathogen. The inhibition of biofilm was also visualized by microscopic observations. The CFS from the isolate tentatively identified as *Streptococcus Faeecalis* obtained from Bifilac was observed to have maximum inhibitory activity (~60%) to disrupt the mature biofilm formed by *C.sakazakii.* The results indicate the strain specific response towards inhibiton of biofilm formed by *C.sakazakii*

From this study we can conclude that commercially available probiotics if taken regularly in adequate amount can be crucial in avoiding the risk of diseases state by increasing the immunity and hence can help to promote the healthy lifestyle to individual. Probiotics effectiveness has been shown against *Cronobacter sakazakii* in this study, which shows that these can be taken for purpose other than treating diarrheal cases. Probiotics intake includes negligible side effects as compared to antibiotics and can be administered daily as a preventive measure to avoid diseases condition in future.[34] Hence from the study we can conclude that Antimicrobial activity of probiotics shows that these can be taken as health supplement on daily basis.

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