SYNERGISTIC EFFECTS OF PREBIOTICS ON PROBIOTICS

Thesis submitted to the Degree of Doctor of Philosophy (Science)

In

Microbiology

By

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ABBREVIATIONS

µl	microlitre
ml	mililitre
l	litre
µm	micrometer
µg	microgram
ng	nanogram
mg	milligram
gm	gram
SCFA	Short chain fatty acid
SEM	Scanning electron microscope
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
BSA	Bovine serum albumin
CAT	Catalase
CCl_4	Carbon tetrachloride
HPLC	High performance liquid chromatography
Spm	Spermine
Spd	Spermidine
TAA	Thioacetamide
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TBARS	Thiobarbituric acid reactive substances
MDA	Malonaldehyde
dH_2O	Distilled water

Contents

ABSTRACT

Aim: This study was aimed to evaluate the effects of prebiotics viz fructo-oligosaccharide, inulin and maltodextrin on the growth of probiotic yeast *Saccharomyces boulardii* and three probiotic lactobacilli *Lactobacillus casei, Lactobacillus rhamnosus* and *Lactobacillus plantarum.* Based on the in vitro results, the synbiotic effect of *S.boulardii* and inulin was evaluated on mouse gut microbiota by analyzing stool samples. Interaction between *S.boulardii* and common gut bacteria was studied in vitro in presence of different prebiotics. Effect of *S.boulardii* was checked on liver polyamines and damaged liver in mouse model. Effects of the abovesaid prebiotics was checked on the growth of lactobacilli in presence of antibiotics.

Results**:** *S.boulardii* was found to grow better in presence of inulin in vitro. Administration of inulin and *S.boulardii* together altered the number and nature of microorganisms in the stool sample of mice. *In vitro* association studies revealed that *S.boulardii* grown in inulin could interact and form large aggregates with *E.coli* and *E.faecalis.* Aggregation is dependent on time and did not form in presence of other prebiotics or other bacteria like *S.aureus* or lactobacilli. The association is a property of the live *S.boulardii* as heat treatment and translation inhibitor, cycloheximide completely abolished the aggregate formation.

The prebiotics increased the growth of the lactobacilli though the sensitivities of the lactobacilli to antibiotics was found to vary with the prebiotic. Maltodextrin was found to change the viability of *L.casei* and made it sensitive towards azithromycin while had very little effect on other two lactobacilli. *L.casei* cells became shorter and lost their viability. *L. plantarum* became sensitive towards ampicilin and ciprofloxacin in the presence of maltodextrin and inulin respectively compared to dextrose as revealed by MTT assay and fluorescence microscopy whereas sensitivity of *L. rhamnosus* remains the same.

Oral administration of *S.boulardii* was found to have hepatoprotective role against CCl_4. All the markers of hepatotoxicity were found to decrease in the serum of mice treated with *S.boulardii* prior to CCl_4 administration. The concentrations of polyamines, known as hepatoprotectives, were found at increased level in mice liver treated with *S.boulardii*.

Conclusion: Aggregate formation between *S.boulardii* and *E.coli/E.faecalis* in presence of inulin and their subsequent removal might be the reason for change in composition of microbes in stool in mice. As the sensitivity of lactobacilli towards antibiotics changes in presence of prebiotics, proper choice of both for better synbiotic effect is very important for any treatment.

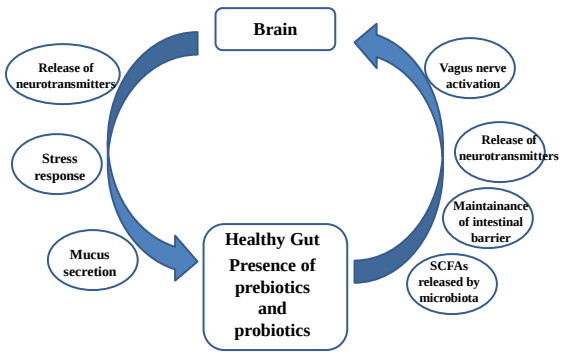
CHAPTER 1

General Introduction, Aims and Objectives

1. INTRODUCTION

Our gut environment is strongly affected by the diet and can immensely influence the quality of life of us. The submucosa, the layer beneath the gut epithelial layer, harbours more than 100 million nerve cells, called the enteric nervous system (ENS). ENS is connected to our central nervous system (CNS). The cross talks between the gut microbiota, gut tissue, ENS and CNS dictate our digestion, mood, thinking ability and many more things. Hence, more research should be done on the understanding of the physiological, cellular and molecular basis of enteric microbiome-gut-brain communication.

Human microbiome project has revealed the presence of nearly one thousand different types of microorganisms in human gut, which play an important role in the overall health of an individual. The good microorganisms having beneficial roles are called probiotics and provide numerous health benefits like immune boost up, improvement of digestive system etc. Probiotics have been found to grow better in presence of short chain oligosaccharides called prebiotics. The combined effect of both probiotics and prebiotics called synbiotics has been found to have more beneficial effects than the individual ones. Hence, extensive research is going on to elucidate the role of synbiotics on various health problems and on the modulation of gut microbiota composition.



Combination of Pre- and Probiotics has more beneficial effects than individual components

1.1 PROBIOTICS

A healthy gut should have two groups of beneficial bacteria, the Bacteroidetes and the Firmicutes. The phylum Bacteroidetes contain many genera of Gram-negative, non-spore forming, anaerobic or aerobic, and rod-shaped bacteria. Some important genera include *Alistipes*, *Bacteroides*, *Parabacteroides*, *Prevotella*, *Paraprevotella*, *Alloprevotella*, *Barnesiella*, *Tannerella* etc. These microorganisms are widely distributed in the environment including soil, water etc. They are abundant in intestine reaching up to 10^{11} cells/g of intestinal material and in symbiotic relationship with the host. The other major phylum present in our gut is Firmicutes which contains mostly Gram +ve bacteria. Some important classes are Bacilli, Clostridia, Erysipelotrichia etc.

Probiotics are live microorganisms that have numerous beneficial effects on the host animal. The probiotic microorganisms used for pharmacological purposes are usually lactic acid producers such as Lactobacilli and Bifidobacteria and non-pathogenic yeast like *Saccharomyces boulardii*. Lactobacilli belong to the phylum Firmicutes and order Lactobacillales and Bifidobacteria belong to the phylum Actinobacteria and order Bifidobacteriales. *Saccharomyces boulardii,* a species of tropical yeast, belongs to the class Saccharomycetes and order Saccharomycetales. These microorganisms are usually non-pathogenic to the host, resistant to gastric juices, able to adhere to the cells of intestinal epithelium layer and show antagonistic activity to the enteric pathogens. The use of probiotics in the form of fermented food was very common since the early 20th century. Long time back, Elie Metchinkoff, who is considered the 'father' of probiotics, proposed the "beneficial effects of the bacteria present in yoghurt and attributed the long life of Bulgarian peasants to their regular intake of yoghurt containing *Lactobacillus* species". Presently a considerable number of pharmaceutical preparations of probiotics like lactic acid bacteria and yeast are available in the form of capsules, tablets, lyophilized powder etc. in medicine shops.

Probiotic lactobacilli: *Lactobacillus acidophilus*, *Lactobacillus plantarum, Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus brevis, Lactobacillus delbreuckii subspecies bulgaricus*, *Lactobacillus reuteri*, *Lactobacillus fermentum* etc.

Probiotic Bifidobacteria: *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Bifidobcterium catenulatum* etc.

Other probiotic organisms: *Streptococcus salivarius ssp. thermophilus***,** *Lactococcus lactis ssp. lactis***,** *Lactococcus lactis ssp. cremoris***,** *Enterococcus faecium***,** *Leuconostoc mesenteroides ssp. dextranicum***,** *Propionibacterium freudenreichii***,** *Pediococcus acidilactici***,** *L. gasseri*, *L. confuses.*

Probiotic yeast: *Saccharomyces boulardii*

1.2 FUNCTIONS OF PROBIOTICS:

Probiotics have been found to show numerous health benefits like

i) Lowering of cholesterol:

In vitro and in vivo results have supported the potential of probiotics in improvement of serum lipid profile by removing cholesterol (Min-Tze Liong et al, 2015). Probiotics have been found to assimilate cholesterol on cellular surface and into cell membrane. They also help in coprecipitation of cholesterol with deconjugated bile produced by bile hydrolase and production of short-chain fatty acids. Probiotics have been found to perform dual roles in cholesterol metabolim. One hand, probiotics have been found to lower the cholesterol level in blood by deconjugation of bile. On the other hand, these deconjugated bile acids repress the synthesis of bile acids from cholesterol. It has been demonstrated that deconjugated bile acids bind to nuclear receptors namely the farsenoid X receptor (FXR) with higher affinity and lead to reduced transcription of cholesterol 7-alpha hydroxylase (7AH) gene. This enzyme is responsible for bile acid synthesis from cholesterol and reduced expression leads to improper absorption of lipids, formation of gallstones, carcinogenesis in colon and other varying gut diseases. Probiotics together with prebiotics also prevent the formation of abnormal erythrocytes caused by hypercholesterolemia, improve the membrane fluidity of erythrocytes, decrease rigidity of erythrocyte membrane and alter composition of membrane lipids. Hence, probiotics and prebiotics can be considered as a new feasible and natural intervention for cholesterol management (Anderson et al, 1999).

ii) Reducing the blood pressure

Several studies have established that dietary polyphenols play a positive role in the reduction of cardiovascular complications by reducing blood pressure. Probiotics interact with polyphenols and control their bioavalability and thus has a role in lowering of blood pressure. This has been proved clinically though there is no confirmative link between intake of polyphenols/probiotics and improvement of Arterial hypertension has been achieved yet (Liong et al, 2007; Lye et al 2009; Kearney et al, 2005).

iii) Treating rheumatoid arthritis- Rheumatoid arthritis (RA) is one of the common autoimmune problems suffered by men and women nowadays. The role of dietary modifications in RA is not well understood though some benefits of vegeterian diet can be explained by antioxidant constituents. It has been found that gut microbiota is altered in RA and probiotics can help to alleviate RA symptoms by

modulating gut microbiota. It has been found that supplementation of *L. casei* 01 improved the disease activity and inflammatory status of patients with RA. The levels of three serum proinflammatory cytokines i.e, tumor necrosis factor-α, interleukin-6, and interleukin-12 were decreased significantly after treatment with probiotics. The level of interleukin-1 β was not altered significantly by the probiotics. The serum level of regulatory cytokine (interleukin-10) and the ratio of interleukin-10 to interleukin-12 were significantly increased in the probiotic treatment (Elnaz et al, 2014).

iv) Reduction in the risk of certain cancers

In additional to their conventional use as gut modulators, role of probiotics has been investigated for prevention of cancer. Several in vivo and molecular studies have shown that many probiotics have antimicrobial effect against carcinogen-producing microorganisms. They might also play role as antimutagenic and take part in the alteration of the tumor differentiation processes (Collins et al 2006) though more studies are required before coming to any conclusion.

v) Alleviation of symptoms of lactose intolerance

Lactose intolerance, a very common phenomenon observed in a large proportion of the world's population. It is the inability to utilise lactose because of the lacking of the enzyme lactase, which is present in suckling infants but disappears after weaning. Problems appear if people with Lactose intolerance migrate to other countries having considerable amount of lactose in some form of food, then it becomes difficult to avoid (Oak et al, 2019).

As most of the lactose is converted to lactic acid in yoghurt by LAB, people face hardly any problem in consuming it. Sometimes yoghurt is supplied with preformed lactase that converts the lactose in the small intestine. Regular use of yoghurt has been found to restore the activity of lactase in the intestine of children with *Giardia lamblia* infection.

vi) Prevention or reduction of the effects of atopic dermatitis

In recent studies, probiotics have been found to prevent and reduce the symptoms of allergy by interfering with immunomodulatory responses. In a study, BALB/c mice were fed with a mixture of four species of probiotics, *Bifidobacterium lactis*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus plantarum*, together with sodium butyrate to find out their combinatorial effects on allergic symptoms. The treatment was found to reduce thicknesses of ears, the quantity of leaked Evans blue, a measure of vascular permeability, serum histamine levels etc. The treatment increased the levels of serum IL-10, an

anti-inflammatory cytokine. Differentiation of Th1 and Treg cells increased in mesenteric lymph node and spleen. The ratio of Firmicutes/Bacteroidetes changes in the gut suggesting the possible use of probiotics and sodium butyrate in removing allergic symptoms in atopic dermatitis (Huang et al, 2017).

vii) Curing of Crohn's disease. Crohn's disease has been found to alter the gut microbiota in patients (Didari et al 2015). It has been seen that application of *Saccharomyces boulardii* along with standard therapy can increase clinical benefit more than the use of probioics alone. On the contrary, the probiotic mixture VSL#3 has proved to be effective in preventing relapses of active inflammation after successful antibiotic treatment. More studies are needed to better identify the exact role of probiotics in Crohn's disease (Prantera et al, 2006).

viii) Action against diarrhea- Sometimes life threatening diarrhea and inflammation in colon is caused by *C. difficle* bacteria and leads to colitis. Regular use of probiotics helps to prevent colonization of these bacteria (Kotowska et al, 2005).

ix) Relieve constipation and irritable bowel syndrome- According to the recent findings, altered nature and number of gut microbiota, referred as dysbiosis, plays an important factor in the pathogenesis of irritable bowel syndrome (IBS). Several studies have indicated that probiotics can be used to manipulate or alter the gut microbiota. It is therefore necessary to evaluate their role in the management of the global IBS symptoms. Together with prebiotics, probiotics can reduce abdominal pain, bloating and flatulence in patients with IBS and changes stool consistency. Hence, probiotics can be used in treatment of IBS, particularly where diarrhea predominants (Didari et al, 2015).

x) Prevention of candidiasis and urinary tract infections (UTI)

Candidiasis, a type of infection, is caused by more than 20 different species of yeasts belong to the genus *Candida*. Oral candidiasis or thrush with white patches on tongue is very common. Other common candidiasis includes skin infections and vaginal yeast infection. Overgrowth of some species of Candida can lead to systemic infections called invasive Candidiasis.

Several *Lactobacilli* strains have been screened and found to have considerable differences in efficacy against *Candida*. In one study, 30 lactobacilli strains were tested against three strains of *C. albicans.* Three strains of lactobacillus, *L. paracasei 28.4, L. rhamnosus 5.2* and *L. fermentum 20.4* showed great antifungal activity by preventing the formation of biofilm. (Rossoni et al, 2018). The peptide toxins produced by lactobacilli e.g, bacteriocin L23 produced by *L. fermentum L23* (Pascual et al, 2008),

plantaricin produced by *L. plantarum* (Sharma et al, 2014); pentocin TV35b produced by *L. pentosus* (Okkers et al, 1999) are effective against Candida. Many low molecular weight substances are produced by lactobacilli like reuterin, reutericyclin and dyacetyl (Talarico et al, 1988; Jay et al, 2012) which are effective against Candida.

Probiotics have been found to prevent and treat recurrent complicated and uncomplicated urinary tract infections (UTI) and it can act as a promising alternative to conventional medicines.

xi) **Enhancement of specific and nonspecific immune response-** It is postulated that breast fed infants possesses higher health advantage over formula fed infants due to an elevated Bifidobacteria count. Probiotic bacteria inhibit growth of pathogens by lactic acid, butyric acid and bacteriocins. These substances and an alteration is found in the ecological balance of enteric commensals. Improvement in erythrocyte membrane fluidity and reduction of membrane rigidity and alteration in membrane lipid profile can be mediated by the administration of prebiotics and probiotics (Galdeano et al, 2019).

xii) Maintainance of Oral health

The natural microbial balance in the oral cavity is extremely important for maintaining oral health and this new insight has brought a new concept to dentistry. An antagonistic role of probiotic lactobacilli and bifidobacteria has been found against salivary *Streptococcus mutans,* the bacteria responsible for tooth decay (Shiva et al. 2012; Jørgensen et al, 2016). Use of probiotics has shown to reduce dental caries incidence and arrest of root caries (Hedayati et al, 2015) and improvement in the standard markers like plaque index, gingival index, probing depth have been found in gingivitis and periodontitis. Changes have been also found in subgingival microbiota and pro-inflammatory cytokine levels in gingival crevicular fluid upon administration of probiotics. Hence, probiotics can be used along with standard treatment for oral health though the local and systemic mechanisms of actions are still largely unknown. More research should be conducted before its clinical recommendation.

1.3 Possible mechanism of action of probiotics

Researchers are trying their best to find out the mechanism of action of the probiotics. It has been found that there are few ways by which they might be operating. They include

i. Strengthening of the epithelial barrier in gastro-intestinal tract
ii. Adhesion to intestinal mucosa
iii. Competitive inhibition of pathogenic microorganisms
iv. Synthesis of anti-microbial substances

v. Improvement of the immune system.

i. Strengthening of the epithelial barrier in gastro-intestinal tract

Probiotics adhered to the intestinal layer or mucous membrane prevent the binding and entry of pathogenic microorganisms through these layers (Servin.et al, 2003). Probiotics also compete for limited resources available for growth with other microorganisms. Probiotic strain *E. coli* Nissle 1917 possesses multiple iron uptake mechanisms, which provides competitive inhibition of the growth of other intestinal microbes and pathogens (Große et al 2006). Maintainance of proper intestinal barrier is essential for its function, which is maintained by tight junctions between the cells. Several other factors like mucus production, secretion of water and chloride are also required for structure function maiintanance of gut epithelium. Loss of membrane integrity is seen in several disease conditions including infectious diarrhea (Sakaguchi et al, 2002), inflammatory bowel disease (Schmitz H et al, 1999, Wyatt et al, 1993) and autoimmune diseases including type 1 diabetes mellitus (Watts et al, 2005). Probiotics benefit the host in these diseases improving gut epithelium health. Studies have shown Lactobacillus species improves barrier integrity, prevents development of colitis in mice deficient in interleukin 10 (Madsen et al, 1999).

ii. Adhesion to intestinal mucosa

One of the main criteria for the selection of new probiotic is their adhesion property to gut epithelium (Juntunen et al, 2001; Schiffrin et al, 1997). Several surface determinants have been found on the surface of LABs which are involved in the interaction with the membranes. Probiotics should not be washed off by mucin, the complex glycoprotein present in mucous, which prevents the adhesion of pathogenic bacteria (Collado et al, 2006; González et al, 2012). This interaction is also important for modulation of the immune system (Perdigon et al, 2002) and antagonism against pathogens (Hirano et al, 2003) (Salminen et al, 1998; Crociani et al, 1995). This process is also mediated by saccharide moieties and lipoteichoic acids (Vélezl et al, 2007). The mucus-binding protein (MUB) produced by *Lactobacillu reuteri* is the most important example of mucus-targeting bacterial adhesions (Colladois et al, 2005). Involvement of surface proteins has been established between human plasminogen or enterocytes and *Bifidobacterium animalis* subsp. *lactis* (Candela et al, 2007, 2009; Sánchez et al, 2010). Another protein, mucous adhesion-promoting protein (MapA) is responsible for mediating the binding of *L. reuteri* and *L. fermentum* to mucus membrane (Ouwehand et al, 2002). Two mucins, MUC2 and MUC3, are produced by *L. plantarum* which inhibit the adherence of enteropathogenic *E. coli* to the mucus layer. These observations indicate that modulating the mucin secretion and thus the mucous layers and glycocalyx overlying the intestinal epithelium, probiotics provide protection against invasion by pathogens (Hirano et

al, 2003; Voltan et al, 2007). Comparison of the adhesion property of acid resistant *Bifidobacterium longum* and *Bifidobacterium catenulatum* strains to the acid sensitive strains to human gut showed that "in half of the 4 studied cases, the acid-resistant derivative showed a greater ability to adhere to human intestinal mucus than the original strain" (Collado et al, 2006). The mixture of probiotics in VSL3 has been reported to increase the expression of mucin gene depending on the adhesion of bacterial cells to the intestinal epithelium (Caballero et al, 2007; Kim et al, 2010).

Presence of specific strains of some probiotics can induce the release of small peptides from the epithelial cells. These peptides are called defensins that form pores on the membrane and kill the bacteria, fungi and viruses and stabilize the gut barrier function (Furrie et al, 2005; Kagan et al, 1990). Paneth cells secrete various enzymes that attack the bacterial membranes like lysozyme hydrolyzes the glycosidic linkage of wall peptidoglycan (Müller et al 2005) and phospholipase A degrades the bacterial membrane phospholipids (Koprivnjak et al, 2002). Cathelicidins, a group of cationic helical peptides, bind to bacterial membranes through electrostatic interactions and kill the pathogenic microorganisms (Bals et al, 2003). More studies are required to study the interactions between the probiotics and the gut membrane and subsequent elimination of non-beneficial microorganisms (Kim et al, 2010).

iii. Competitive inhibition of pathogenic microorganisms

In 1969, Greenberg reported the total exclusion of *Salmonella typhimurium* from maggots of blowflies and first used the term 'competitive exclusion' for the phenomenon 'where one species of bacteria vigorously competes for receptor sites with another species in the intestinal tract' (Greenberg et al, 1969). A number of different mechanisms are involved behind this phenomenon (Rolfe et al, 1991).

a) Competition for adhesion receptors- Interaction between gut epithelial cell surface proteins, mucins and probiotics make the receptors unaccessible to the pathogenic bacteria and inhibit their colonization (Servin et al, 2004). Lactobacilli and bifidobacteria have been found to inhibit several pathogens, e.g *E. coli*, *Salmonella sp*, *Helicobacter pylori sp*, *Listeria monocytogenes* and *Rotavirus* (Chenoll et al, 2011; Sgouras et al, 2004; Todoriki et al, 2001).

It has been observed that bacteria usually modify their environment to inhibit the growth of its competitor and facilitate its growth. One such way is by the production of lactic and acetic acid, which modifies the microenvironment (Schiffrin et al, 2002). Some lactobacilli and bifidobacteria have been found to share specific carbohydrate moieties with some enteropathogens (Nesser et al, 2000), making the strains compete for the receptors on host cells (Mukai et al, 2002). Competitive exclusion of pathogens by

probiotics has been demonstrated in vitro (Tuomola et al, 1999; Ouwehand et al, 1998) and (Hirn et al, 1992) in vivo using checken and pig mucosal material (Genovese et al, 2000; Hirano et al, 2003).

b) Competition for nutrients- There is always a competition for food in the gut among the gut resident microbes. It is evident that intestinal microbiota compete more efficiently for glucose, N-acetyl-glucosamine, sialic acid etc than *C. difficile* and leads to competetive exclusion of pathogens.

iv. Synthesis of anti-microbial substances

Antimicrobial compounds produced by lactic acid bacteria (LAB) give them a competitive advantage over other microorganisms. The efficacy and spectrum of antimicrobial products of LAB are broad and include low molecular weight molecules (1,000 Da), like lactic acid, acetic acid and other organic acids, hydrogen peroxide, carbon dioxide, diacetyl as well as bacteriocins or bacteriocin-like substances (Mishra et al, 1996). Acetic acid and lactic acid, are the two main antimicrobial compounds that act against Gram –ve bacteria (Alakomi et al, 2000; De Keersmaecker et al, 2006; Makras et al, 2006) The dissociation of organic acids inside the bacterial lead to lowering of the pH and accumulation of the ionized form of the acids and lead to the death of the pathogens (Ouwehand et al, 1998; Russell et al, 1998).

Bacteriocins or small antimicrobial peptides are produced by many Gram +ve bacteria which act against many food-borne pathogens. *L. acidophilus* produces lactacin B while *L. plantarum* and *Lactococcus lactis* produces plantaricin and nisin respectively (Nielsen et al, 2010). Bacteriocins are small peptides which form pores in the target cell membrane and cause lysis of them. Another way of killing is by inhibition of cell wall synthesis in spore-forming bacilli (Hassan et al, 2012) (Bierbaum et al, 2009). Bacteriocin production may enable the direct inhibition of pathogen growth within the gastrointestinal tract (O'Shea et al, 2012).

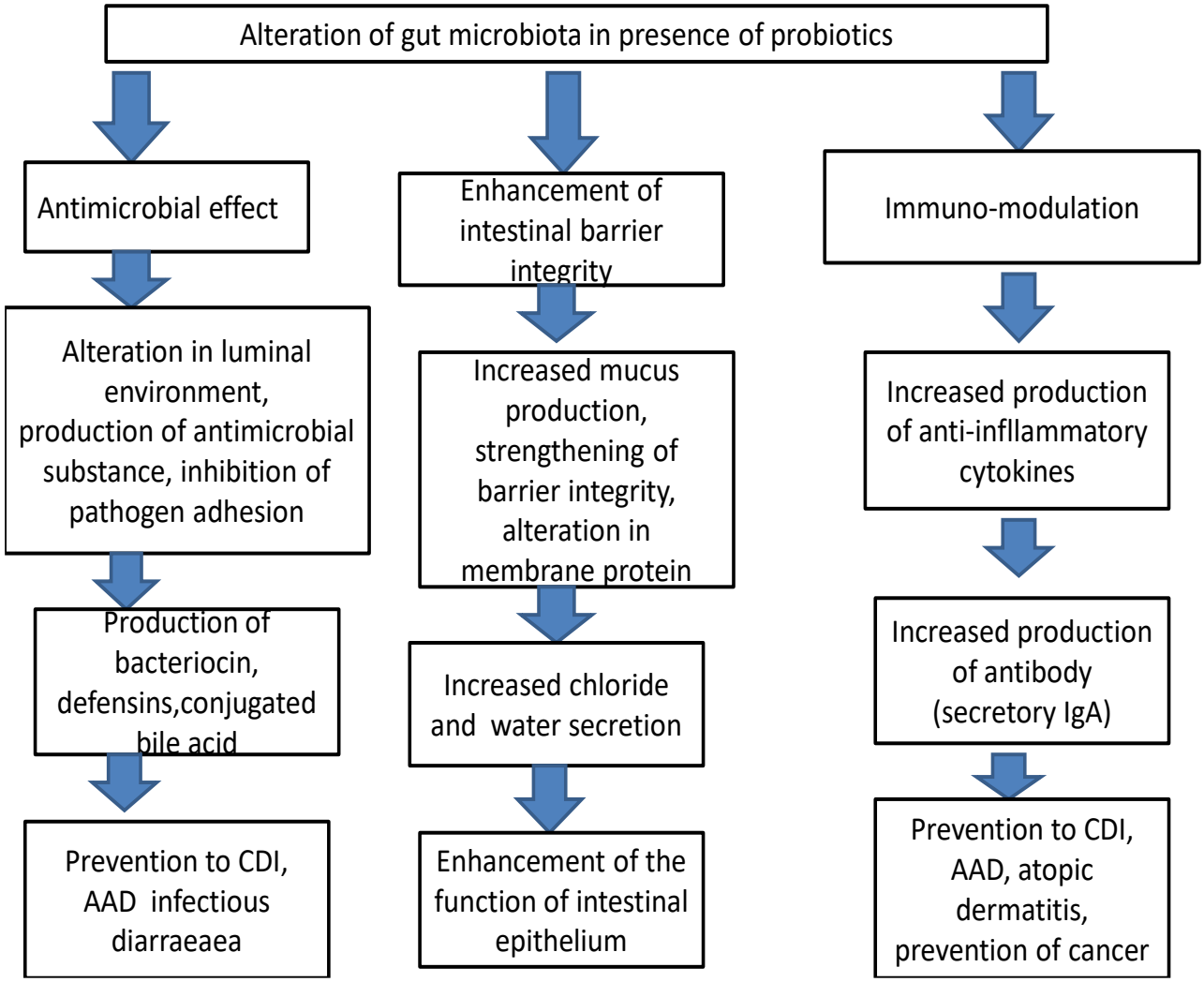


Figure 1: Effects of probiotics on gut microbiota

v. Improvement of the immune system.

Probiotic bacteria have immunomodulatory effect as they can interact with dendritic cells (DCs), monocytes/macrophages, lymphocytes, regulatory T (Treg) cells, immunoglobulin A (IgA)–producing B cells, natural killer cells etc. Probiotics have been found to induce T cell apoptosis. The primary response to pathogens is triggered by pattern recognition receptors (PPRs) like toll-like receptors (TLRs) which bind to pathogen associated molecular patterns (PAMPs) present on pathogen surface (Lebeer et al, 2010). The interaction between probiotics and intestinal epithelial cells (IECs) or DCs is through the PPRs present on the microbes (O'Shea et al, 2012; Lebeer et al, 2010).

1.4 Probiotics used in the study

The work presented in this thesis involves the probiotic bacteria *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus casei* and probiotic yeast *Saccharomycs boulardii.*

Scientific classification of Lactobacillus sp (Bijerinck 1901)

Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	*Lactobacillus*
Species	*L. casei, L.acidophilus, L.rhamnosus, L.plantarum*

1.4.1 *Lactobacillus plantarum*

Lactobacillus plantarum, a common member of the genus *Lactobacillus*, is used to make many fermented food products like sourdough bread, sauerkraut, kimchi, olive brines, Nigerian ogi, fufu, as well as in anaerobic plant matter. It is Gram +ve, bacillus with rounded ends and found in single, pairs or in short chains. Each bacterium is 3–8 μm long with 0.9–1.2 μm width. *L. plantarum* survives the stomach acid easily and the optimum growth temperature is close to human body temperature.

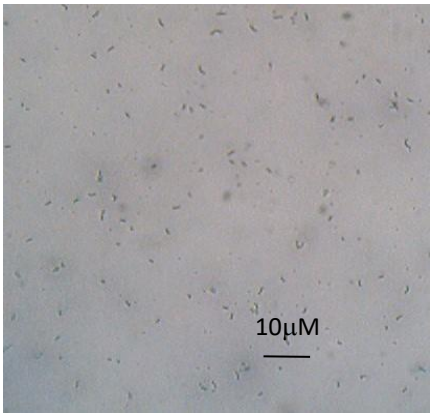


Figure 2: *Lactobacillus plantarum* as seen under light microscope after methylene blue staining (40x)

Functions:

- *L. plantarum* significantly reduces the symptoms of irritable bowel syndrome (IBS) by suppressing the growth of gas-producing bacteria in the intestines (Bixquert et al, 2009).
- *L. plantarum* helps to maintain the intestinal permeability and has been found to have significant antioxidant activities. It fights against various intestinal pathogens for example *C. difficile*. The antimicrobial substances produced by *L. plantarum* have profound effects on both Gram +ve and –ve vacteria and help the probiotic to survive in the gut (Bested et al, 2013).
- *L. plantarum* has also been found to fight burn infections (topical), strengthen our immune system, decrease frequency and duration of flu, reduce the risk of pneumonia etc. It has been also found to reduce sepsis of pancreas, infections related to postoperation, lowers the risk of kidney stone, reduce blood pressure. *L. plantarum* also decreases inflammatory response by producing more Th2 and decreasing the levels of Th1/Th2.
- *L. plantarum* makes lysine, an essential amino acid (Landete et al, 2010).

Side effects of *Lactobacillus plantarum* on the host

L. plantarum has very mild side effects on its host which includes mild digestive discomfort in some.

1.4.2 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus, one of the non-colonizing transient bacteria of our gut, is the probiotic of choice for its beneficial effects on digestive system.

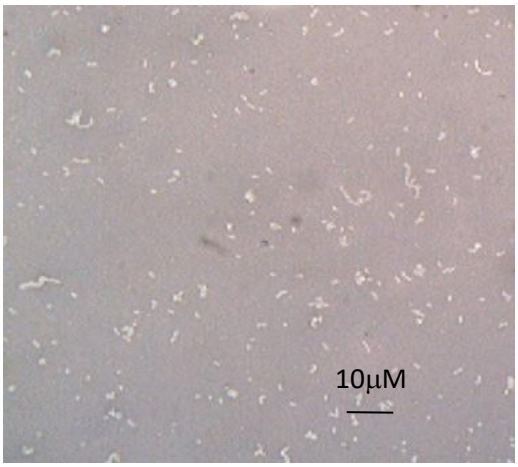


Figure 3: Negative staining of *Lactobacillus rhamnosus* under light microscope (40X)

Functions:

L. rhamnosus has huge research importance as it possess a positive impact on gut health, as well neurological function. *L. rhamnosus* is added in probiotic supplements and found in fermented foods like sauerkraut etc. *L. rhamnosus* is an immensely beneficial non-colonizing LAB which is used to treat leaky gut syndrome, maintain a balance in the gut health. It also improves neural function and maintains proper neurological health. *L. rhamnosus* has been found to fight against many common bacterial and fungal infections such as *Candida albicans.* (Allonsius et al, 2019).

Importance in gut health

L. rhamnosus was discovered in 1983 in the intestines of a healthy human. In contrary to other *Lactobacillus* strains, *L. rhamnosus* is considered a transient strain as it is unlikely to be present in the GI tract without regular consumption. Hence, *L. rhamnosus* supplements are vital for maintaining the overall gut health and should be taken regularly. Several reports showed that administration of *L. rhamnosus* in children suffering from irritable bowel syndrome (IBS), improves associated symptoms like abdominal pain and loose motion and frequent bowel movement significantly (Pederson et al, 2014).

Prevention and cure of Candidiasis

Overgrowth of *Candida* called Candidiasis is very common among women though men are also susceptible to it. Candida overgrowth though very common in tongue and vagina as seen as a white coating, has also been found in the small intestines and lead to leaky gut syndrome, sensitivities to food etc. Chronic infection by candida results in different negative physical and mental conditions and fatigue syndrome. *L. rhamnosus* has beneficial effects in the treatment candidiasis especially for *Vulvovaginal candidiasis*, the common vaginal yeast infection. *L. rhamnosus* together with other probiotics and prescribed anti-fungal drugs has been shown to inhibit the growth of candida (Allonsius et al, 2019). Preliminary studies and results on *L. rhamnosus* are promising in addressing many important medical concerns like food allergies & intolerances, diarrhea, respiratory tract infections, eczema, UTIs and obesity.

Potential Risks

In a very few cases, *L. rhamnosus* has been known to impact health conditions negatively.

1.4.3. *Lactobacillus casei*

Lactobacillus casei, the Gram +ve, non-motile and non-spore-forming facultative anaerobes found in human urinary tract and mouth. The average length of the cells is 2.0-4.0 micrometer with average width of 0.7-1.1 micrometer. Strains of *L. casei* are routinely used as probiotics and synbiotics in dairy and drug industries.

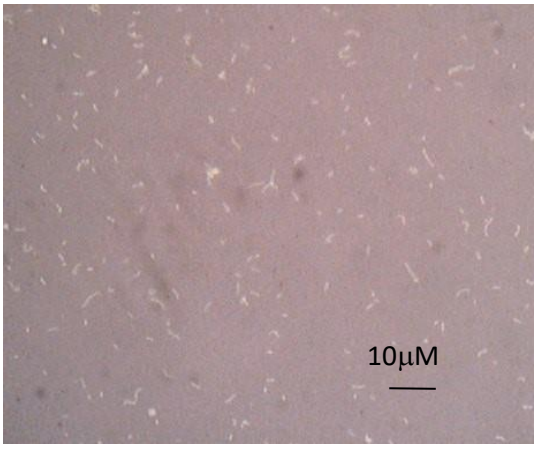


Figure 4: *Lactobacillus casei* as seen under light microscope after negative staining

Functions of *L. casei*:

L. casei is an industrially important probiotic which has huge industrial functions specifically for dairy production. *L. casei* is a nonstarter LAB and used in ripening cheddar cheese and also predominates in naturally fermented Sicilian green olives. (Banks et al, 2004; Randazzo et al, 2004)

In a study, it was found that *L. casei* strain Shirota efficiently inhibits the growth of *Helicobacter pylori* in experimental model while prevented colonization of it only slightly in humans (Cats et al, 2003).

Some *L. casei* strains are effective in reducing pathogenic bacterial diseases in GI tract especially effective against acute and infectious diarrhea. *L. casei* has been combined with other probiotic strains of bacteria in randomized trials and has been found to prevent antibiotic-associated diarrhea (AAD) and *Clostridium difficile* infections (CDI) compared to placebo control (McFarland et al, 2009).

Commercial use:

L. casei DN-114001 and *L. casei* Shirota have been extensively studied among the best-documented probiotics. *L. casei* is widely used in commercially available probiotic supplements like Actimel, Yakult, Danactive etc.

1.4.4. *Saccharomyces boulardii*

The probiotic yeast, *Saccharomyces boulardii* is widely used in many countries to treat diarrhea. The organism was first isolated from the fruit lychee in 1923 by French scientist Henri Boulard. It is effective against acute and chronic diarrhea (Kurugol et al, 2005, Billoo et al, 2006). Recent studies have showed that oral administration of *S. boulardii* for 3 weeks in humans have increased the brush border enzyme activity of the duodenal mucosa. Buts et al showed that “polyamines released from *S. boulardii* in the rat ileum stimulate the maturation and turnover of small intestine enterocytes which in turn increase the recovery rate of a patient from diarrhea” (Buts et al, 1994). *S. boulardii* also helps to recover from Crohn’s disease (Guslandi et al, 2000).

Scientific classification of *Saccharomyces boulardii*	
Kingdom	Fungi
Division	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	Saccharomyces

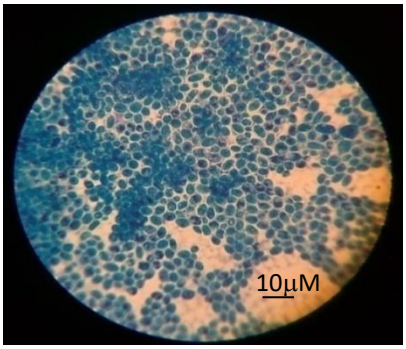


Figure 5: Methylene blue staining of *Saccharomyces boulardii* as seen under light microscope (40X)

S. boulardii has been found to significantly reduce the rate of recurrent *Clostridium difficile* infection by releasing a 54 kDa protease which digests the 'toxin A' molecule secreted by *C. difficile* and its receptors on intestinal brush border cells (Castagliuolo et al, 1999). The pathogenecity of *Escherichia coli* and *Salmonella typhimurium* is also reduced by inhibiting their binding to intestinal brush border cells. In presence of *S. boulardii*, the bacteria bind to the mannose present on the yeast surface by their lectin receptors. It is then eliminated from the body during the next bowel movement (Gedek BR, 1999). *S. boulardii* can survive in pH 3.0-5.0 and resistant to commonly used antibiotics. Administration of *S. boulardii* with beta-lactam antibiotics is highly effective against antibiotic associated diarrhea without any adverse reactions (McFarland et al, 1994, 1995; Kotowska M, 2005). It can be taken at the same time with other probiotics like *Lactobacillus* sp and *Bifidobacterium* sp.

Recent studies have showed the synbiotic use of prebiotics and *Saccharomyces boulardii* as a promising agent towards medical therapy.

1.5 PREBIOTICS

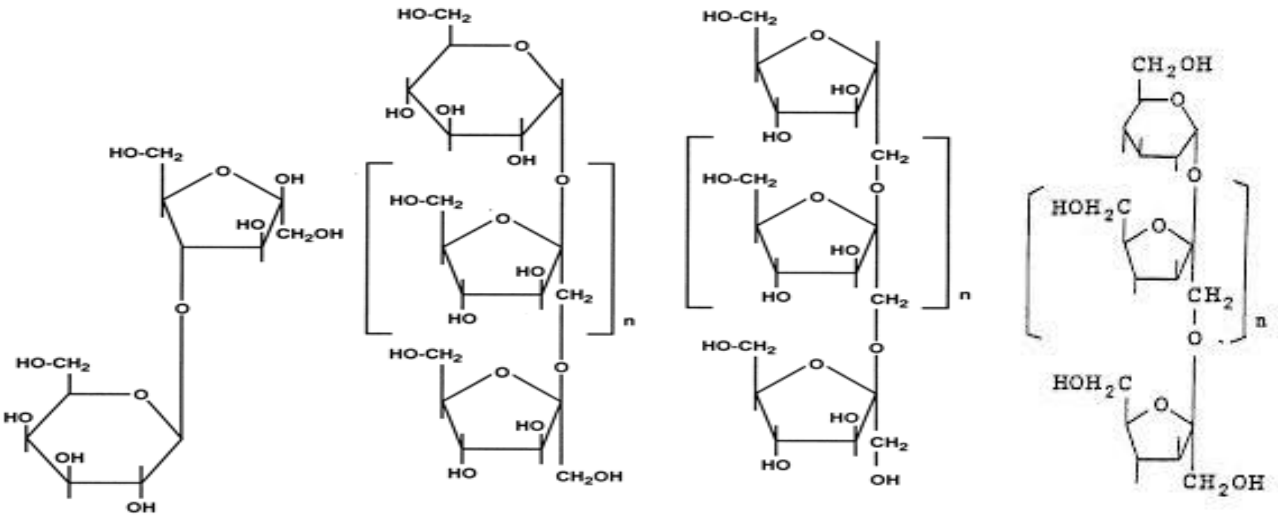
Prebiotics are small oligosaccharides which are nondigestible in the small intestine and travel down to large intestine. A limited number of bacterial species already residing in the colon can act upon them and extract the energy. Thus the prebiotics are used up completely by colonic bacteria and provide more energy to the host. At the same time, the growth of bacteria increases and several by-products are produced which have health benefits to human health.

Examples of Prebiotics

Fructooligosaccharide, Glucooligosaccharides, maltodextrin, inulin etc

Natural sources: Garlic, onions chicory root, Asparagus, whole wheat, rye, barley

Figure 6: Natural sources of prebiotics (Figure taken from www.google.com)



Lactulose **Inulin** **Fructooligosaccharide** **Maltodextrin**

1.5.1 Functions of prebiotics and their impacts on human health

In the large intestine, Prebiotics are utilized or fermented very selectively by probiotics like Bifidobacteria, Lactobacilli to produce lactic acid as main product and some beneficial by-products like short-chain fatty acids (SCFAs), acetate, propionate and butyrate etc. The acids produced lower the pH of intestine and hence kill the harmful organisms. They also act as signaling molecules on specific receptors. Butyrate has been found to maintain gut epithelial membrane homeostasis. Applications of both pre and probiotics together have been found beneficial on several diseases and disorders.

Till date, most searches for prebiotics are directed toward the growth of lactic acid producing microorganisms. Inulin have been found to cause 5 and 10 fold increase in total anaerobes and Lactobacilli respectively when added in medium having the condition of colon. Galacto-oligosaccharide (GOS) addition showed increase in Lactobacilli count (Mcbain and Macfarlane, 2001).

1.6 SYNBIOTICS

Probiotics and Prebiotics: Current Status and Future Trends

Applications of both pre and probiotics together have been found beneficial on several diseases and disorders. The combination of both prebiotics and probiotics could improve the survival of the probiotic organism since the prebiotic supplies the specific substrate for its fermentation and result in advantages to the host (Collins et al, 1999).

In last few decades research on effects of probiotics and prebiotics has enriched our knowledge immensely though care should be taken in application of these agents as effects of probiotics are strain specific and not all strains are beneficial all the time. Reports from different works as well as our own work have shown that prebiotics can have completely opposite effects on two probiotic strains (Venema et al, 2015). Similarly, all prebiotics are not having similar functions and they stimulate the growth of different microorganisms. The action of prebiotics is also dependent on their dose and the culture condition of the probiotics. The mechanism of action of the synbiotics is not completely known to the researchers yet and extensive studies are required for better understanding.

1.6.1 Function

The synbiotic products are found to have more antimicrobial qualities, anticarcinogenic qualities, antidiarrheal aspects, antiallergenic qualities etc if compared to the probiotic organisms and prebiotic molecules separately. Synbiotics also reduce concentrations of fat and sugars in blood, modulate immune responses by enhancing innate immune reactions like increasing phagocyte activity of the phagocytes.

Recent studies have showed the synbiotic use of prebiotics and *S. boulardii* as a promising agent towards medical therapy. In 1991, Mitterdorfer et al showed the effect of different prebiotics like galacto-oligosaccharide, inulin, fructo-oligosaccharide and guar gum hydrolysate powder on the growth of 10 strains of *S. boulardii* isolated from various pharmaceutical products (Mitterdorfer et al, 1991). Based on their findings, they suggested the synbiotic use of fructo-oligosaccharides and *S. boulardii* for better results. In 2005, Martin et al showed the promising effect of regular ingestion of *S. boulardii* and inulin in children colonized by *Helicobacter pylori* (Martin et al, 2005). But the amount of research has been done so far on the synbiotic effect of prebiotics and *S. boulardii* is not adequate and more work should be done before concluding anything.

Strategic use of synbiotics can provide major benefits by reducing health problems, especially those related to Gastro-intestinal tract (Vijaya et al, 2005). Fernandes et al showed that Synbiotics have a role in the reduction on high-sensitivity C-reactive protein. They also showed that the prebiotic inulin could lower inflammation by lowering the levels of interleukin-6 and/or tumor necrosis factor (Fernandes et al, 2017). Synbiotics administration may have a beneficial effect on the postcolectomy gastrointestinal function (George et al, 2016). One of the advantages of using yeasts as probiotics over bacteria is that bacteria may transfer the antibiotic resistance gene to pathogenic bacteria but no such gene transfer can occur between bacteria and yeasts, hence yeasts are safe for use during antibiotic treatment (Martins et al, 2009).

1.7 Liver

Liver is the body's primary detoxification site and a healthy liver neutralizes a wide range of toxins, then either recycles them for use in the body, or excretes them. The ammonia produced during protein metabolism and through the action of propeolytic bacteria is converted to urea which is released as urine. In case of malfunctioning of liver, ammonia can enter the bloodstream and invade the central nervous system, causing many dangerous symptoms. In advanced cases of cirrhosis of the liver, a mild brain dysfunction called minimal hepatic encephalopathy (MHE) may develop (Qing et al, 2004). Hence, reduction in concentration of ammonia is an option for the treatment of MHE and use of probiotics in this case has advantages over antibiotic use since antibiotic will kill the beneficial organisms along with the harmful ones. Recent studies have shown that use of synbiotics help MHE patients to recover by reducing their blood ammonia level by killing the protein digesting microorganisms (Steven et al, 2004). Since *S. boulardii* is known to inhibit a number of protein metabolizing microorganisms in intestine, we can presume that it will also lower the ammonia concentration which will in turn reduce the toxic load of ammonia on the liver and throughout the body, including the brain.

AIMS AND OBJECTIVES OF THE STUDY

AIMS

The aim of this study was to determine the effect of different prebiotic molecules on the growth parameters of probiotic yeast *Saccharomyces boulardii* and lactobacilli. Determination of the synbiotic effects of both the prebiotic molecule and *S. boulardii* on intestinal microflora using mouse models and through *in vitro* studies. Determination of the effects of prebiotics on the sensitivity of *Lactobacillus* strains in presence of antibiotics.

OBJECTIVE

- Comparative analysis of the effects of different prebiotics (Inulin, Maltodextrin, Fructooligosaccharide) on the growth of *S. boulardii*
- In vivo monitoring of the effect of the prebiotic (showing maximum effect on the growth of *S. boulardii* in vitro) and *S. boulardii* on intestinal microflora as estimated from mouse stool.
- Studies on the interaction between *S. boulardii* and common gut microorganisms through *in vitro* studies
- Comparative analysis of the effects of prebiotics on the growth of *Lactobacillus spp.* (*L. casei, L. rhamnosus and L. plantarum*).
- To find out the best synbiotic combination during antibiotic treatments.
- Effect of prebiotics on probiotic cell viability, cellular morphology, cell permeability etc
- Role of *S. boulardii* on carbon tetrachloride induced liver damage
- Study of polyamines in liver regeneration

IDEA OF THE WORK

We have seen that several formulations containing prebiotics and probiotics are available in the medicine shops as over the counter drugs. However, the compositions are to some extent random and just the mixture of some probiotics and prebiotics. Although several studies had been done and still being conducted on the synbiotic effects, the proper beneficial composition of pre- and probiotics which helps to prevent certain ailments as well as to maintain gut microbiome are yet to be discovered. In this context, we should also consider that there might be certain adverse effects of irrational use of pre/probiotics as we are dealing with living microbes and chemicals.

Hence, this study has been designed to understand whether we can use these beneficial microbes for the betterment of our health status and to give some constructive inputs regarding the use of pre- and probiotics together. To enrich the knowledge, some animals (mice) were sacrificed with proper clearance from ethical committee to understand the mechanism. It will be premature to say that we have been successful to get all the answers conclusively, but in this thesis certain aspects have been revealed which we think is worth to give a thought to this issue.

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CHAPTER 2

Role of different prebiotics on the growth of *S. boulardii*

2.1 INTRODUCTION

The non-pathogenic probiotic yeast, *Saccharomyces boulardii,* is widely used in Europe for a long time to treat diarrhea and is known to influence the gut environment strongly. Early reports on *S. boulardii* suggest that it is capable of modulating the immune system, inhibiting the cholera toxin action (Castagliuolo et al, 1999), producing diffusible antagonistic compounds that inhibit pathogenic bacterial growth etc. *S. boulardii* has been reported to adhere to bacteria like *Salmonella typhimurium*, enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* etc (Badea L et al., 2003, Mathur et al, 2005, Caprioli A et al., 2005). This adhesion phenomenon has been clearly demonstrated and visualized *in vitro* in some preliminary reports and thought to be one of the mechanisms by which *S. boulardii* prevent pathogen adhesion to specific receptors on the intestinal epithelium (Cusumano et al., 2009). *S. boulardii* is cleared from the gut very quikly as soon as the supplement is discontinued. In a study, it was shown that lectins that bind to mannose glycan on the surface of *S. boulardii* and irreversibly bind to enteric pathogens can be used to prevent the adhesion of these pathogens to the mucosa membranes of the gut and eliminate them from infected patients (Gedek et al, 1999). In another investigation it had been revealed that lectins can cause intestinal dysfunction and bacterial overgrowth, the sole reason for this is its high binding capacity that causes adherence of enteric bacteria to the mucosal surface (Dolan et al, 2010). The bacterial population that do bind to the surface of a yeast cell and don't adhere to the mucosa membrane of the gut are eliminated by the mucus from the lining the epithelial surface (Kalliomaki et al, 2008). The report published by Dr. Tracey J. Lamb on beneficial effect of *S. boulardii,* suggest that there are mechanisms which are different from immune system modulation (Slavin J et al, 2013).

It is a well-known fact that beneficial effects of probiotics are increased in vitro in the presence of prebiotics. But for the best result, the choice and dose of appropriate prebiotic must be determined as there are indications regarding the major beneficial effects of pre and pro biotics if used strategically. The mode of action of pre- and probiotics is believed to be based largely on compositional and functional modulation of the intestinal microbiota. The composition of intestinal microflora can be modulated by changing the food habit and supplementation of natural bioactive molecules and have been found to reduce risks of colon cancer, one of the major health problems throughout the world. Recent studies have showed the synbiotic use of prebiotics and *Saccharomyces boulardii* as a promising agent towards medical therapy. Mitterdorfer et al showed the effect of different prebiotics like galacto-oligosaccharide, inulin, fructo-

oligosaccharide and guar gum hydrolysate powder on the growth of 10 different strains of *Saccharomyces boulardii* isolated from various pharmaceutical products long time back (Mitterdorfer et al, 2001). Based on their findings, they suggested the synbiotic use of fructo-oligosaccharides and *Saccharomyces boulardii* for the understanding of intestinal micro-ecology. In 2005, Martin et al showed the promising effect of regular ingestion of *Saccharomyces boulardii* and inulin in children colonized by *Helicobacter pylori*. But the amount of research has been done so far on the synbiotic effect of prebiotics and *S. boulardii* is not adequate and more work should be done before coming to a conclusive data on their usage.

2.2 OBJECTIVE

- Standardization of the optimum concentration of the preferred prebiotic on the growth of *S. boulardii*.
- Monitoring of the effect of inulin and *S. boulardii* on gut microbiota as revealed by enumeration of microbes in the feces of mice treated with them.
- Based on the in vivo observations, in vitro studies of the associations between *S. boulardii* and other microorganisms in presence of different prebiotics under phase contrast microscope.
- Dose and time dependent association studies between *S. boulardii* and other bacteria in presence of the preferred prebiotics.
- Studies to find out the possible reasons behind the associations.

2.3 MATERIALS AND METHODS

2.3.1 Growth and other media used in the studies:

All the chemicals used and mentioned in this study are procured from Himedia, India.

For growth assay-MRS (De Man, Rogosa and Sharpe) media with dextrose 1% (v/v), MRS media with Maltodextrin (1X - 16X , where 1X concentration represents 1 gm/100ml of dH_20) as carbon source, MRS media with fructooligosaccharides (FOS) (1X- 16X) as carbon source, MRS media with inulin (1X- 16X) as carbon source, Luria broth (LB media). For aggregation studies- we used MRS media where dextrose was replaced by 0.02 g/ml of inulin, maltodextrin and fructooligosaccharides, M63 minimal salt media (5X stock per litre contains - 10g $(NH_4)_2SO_4$, 68G KH_2PO_4, 2.5mg $FeSO_4.7H_2O$ in 1 liter autoclaved distilled water). To make 1X M63 working solution, the 5X media was diluted to 1X with sterile distilled water and added with sterile solution of 1ml 1M $MgSo_4.7\ H_2O$ and carbon source (0.02 g/ml of inulin and 0.02 g/ml of dextrose).

2.3.2 Microorganisms used in the study:

All the probiotic bacteria were collected from MTCC (The Microbial Type Culture Collection and Gene Bank, India. https://mtccindia.res.in/).

Lactobacillus casei MTCC 1423, *Lactobacillus plantarum* MTCC 4462, *Lactobacillus rhamnosus* MTCC 1423, *Escherichia coli* MTCC 1310, *Enterococcus faecalis* MTCC 2729, *Staphylococcus aureus* MTCC 1144. All the bacteria were maintained in MRS media and incubated at 37ºC for 24 hours.

Saccharomyces boulardii was a gift from Prof. Marcin Lukas Zewicz (Faculty of Chemistry Wroclaw University of Technology, Wroclaw, Poland). The cultures is being maintained by routine sub culturing in Sabouraud Dextrose broth with 1% (v/v) inoculum from an overnight culture and incubated at 30^0C.

2.3.3 Determination of relative growth of *Saccharomyces boulardii:*

The growth response of *S. boulardii* on each prebiotic and its different strength relative to its growth response on dextrose was calculated upto 48hrs. The calculation was performed using the following formula in which the growth on dextrose was considered 100%. Relative growth of *S. boulardii* strain on a prebiotic substrate = (A/B) * 100% where A is the mean OD600 value of a strain on prebiotic substrate and B is the mean OD600 value of the same strain grown on dextrose. In this way the result would reflect the growth of a specific *S. boulardii* strain on particular prebiotic substrates relative to its growth on a dextrose substrate which was fixed at 100%.

2.3.4 *in vivo* monitoring of the effect of inulin and *S. boulardii* on the growth of gut microbiota

Male swiss albino mice were acclimatized in the animal house at Bose Institute, Kolkata under laboratory condition. Young male Swiss albino mice (body weight 20±2gm) were acclimatized under the laboratory condition for two weeks before starting experiments. They were given food and water *ad libitum* and were fasted for 16-18 hrs before performing an experiment. The mice were divided into 4 groups with 6 mice each.

Group1: Normal control (No treatment)
Group2: Mice got the prebiotic inulin at a dose of 1g/kg body weight for 7 days and *S. boulardii* at a concentration of 10^7 cells at a time with food for 7 days.
Group3: Mice got only *S. boulardii* at the same dose
Group 4: Mice got only inulin at the same dose

Statement of animal rights:

The work on mice was conducted at Bose Institute and institutional and national guide for the care and use of laboratory animals was followed. (http://www.jcbose.ac.in/centre-for-translational-animal-research)

2.3.5 Stool sample analysis:

Stool samples were analyzed for counting of microorganisms after 7days and 14 days of treatment. 1g stool sample was mixed in 1ml of sterile water and was analyzed for the number and nature of microorganisms present in them following automated aerobic/anaerobic bacteria and fungus identification technique using the instrument Vitek, Biomereux. This work was done at Medica Superspecialty Hospital, Kolkata, India (Link- http://www.medicahospitals.in/).

2.3.6 in vitro experiments to check the interactions between *Saccharomyces boulardii* and other gut bacteria in presence of prebiotics using phase contrast microscope at 20X after 24 hours -

In this experiment, 200µl log phase culture of *S. boulardii* and *E. coli* from MRS media was taken in an eppendorf tube and fresh MRS media was added to make the volume 2ml. This preparation was incubated at 30°C for different time interval (from 1 hour- 4 hours and 24 hours). This step was repeated for both *E. faecalis* and *S. aureus* then checked under phase contrast microscope. Similar experiments were conducted where dextrose in MRS media was replaced by 0.02 g/ml conc. of inulin, fructooligosaccaride and

maltodextrin respectively. This experiment was performed to check if there is any physical interaction between *S. boulardii* and common gut microflora when supplemented with prebiotics such as inulin, fructooligosaccharides and maltodextrin. Since aggregation took place only in presence of inulin, the amount of aggregation was checked at different concentrations of inulin ranging from 0.02g/ml to 0.12 g/ml. All the experiments were repeated thrice. Co-aggregation formation started appearing from 3 to 4 hours but the best result was found after 24 hours of incubation.

2.3.7 Experiment to examine the viability of the organisms in the aggregation:

The cell viability of *S. boulardii, Escherichia coli* and *Enterococcus faecalis* in the clumps were checked. *S. boulardii* and *E. coli* were mixed together and allowed to interact following the same protocol as expt 2.2.6. Similarly, *S. boulardii* and *E. faecalis* were allowed to interact and clumps were isolated from both the interactions by centrifugation at 5000 rpm for 5 minutes and spread over the MRS plates respectively. The plates were incubated for 24 hours at 30ºC. Different colonies from each plate were taken and observed under the microscope to check the presence of yeast cell and bacterial cell with safranin stain.

2.3.8 To find out physical interactions (if there is any) between *S. boulardii* and common gut microflora when supplemented with maltodextrin or fructooligosaccharides in *in vitro*.

The interaction between *S. boulardii* and *Escherichia coli/Enterococcus faecalis* were checked in presence of maltodextrin and fructooligosaccharides. In this experiment, log phase culture of *S. boulardii* and *E. coli* were taken in MRS media having maltodextrin or fructooligosaccharides as the carbon source and followed as experiment 2.3.6 and checked under phase contrast microscope.

2.3.9 To find out the time course of aggregate formation between *S. boulardii* in inulin and *E. coli*

In this experiment, 200µl log phase culture of *S. boulardii* and *E. coli* were taken from MRS media/prebiotic in an eppendorf tube and added fresh MRS media to make the solution upto 2ml.
This preparation was incubated at 30°C for different time interval (from 1 hour- 4 hours and 24 hours). This step was repeated for both *E. faecalis* and *S. aureus* then checked under phase contrast microscope.

2.3.10 To check the effect of heat on *S. boulardii* prior to association

S. boulardii was heat treated at 70ºC for 1 hour, then incubated with live *E. coli* and *E. faecalis* overnight at 30ºC (Here n = 6). The incubated organisms were then observed under phase contrast microscope. To check if the yeast cells are viable or not, little inoculums from live as well as heat treated *S. boulardii* was spread over MRS agar plate (Here n =6)

2.3.11 Treatment of *S. boulardii* with Cycloheximide (a translational inhibitor) to check the involvement of proteins in the clumping (Phase contrast microscope at 20X)

To understand the role of membrane proteins of *S. boulardii* in the co-aggregation, 5 ml of log phase culture of *S. boulardii* grown for 24hrs was treated with cycloheximide (final concentration 250µ/ml) for 4 hours at 30^0C and then centrifuged at 5000 rpm for 10 minutes. The pellet was collected discarding the supernatant and washed twice with 5 ml autoclaved water, centrifuged at 5000 rpm for 10 minutes. The pellet was then resuspended in fresh inulin media.

To check the aggregation property, 500µl of cycloheximide treated *S. boulardii* was incubated with 500µl of *E. coli* in an eppendorf and incubated for 4 hours (as visible aggregation between untreated *S. boulardii* and bacteria starts to appear at 4hrs) and 24hours respectively at 30°C. The interaction was checked under phase contrast microscope at 20X.

2.3.12 To find out the possibility of biofilm formation as a cause of clumping

Biofilm formation was checked as a reason for the aggregation.

The biofilm media composition: M63 minimal salt media (5X stock per litre contains - 10g $(NH_4)_2SO_4$, 68gm KH_2PO_4, 2.5mg $FeSO_4.7H_2O$ in 1liter autoclaved distilled water). To make 1X M63 working solution, the 5X media was diluted to 1X with sterile distilled water and added with sterile solution of 1ml 1M $MgSO_4$, $7H_2O$ and carbon source (0.02 g/ml of inulin and 0.02 g/ml of dextrose). The composition of 400µl final assay mixture was prepared by combining 100 µl of growth Media (MRS or inulin media as carbon source), 200 µl IX M63, biofilm promoting media (Steve et al., 2011) and 100 µl of microbial culture (*S. boulardii/E. coli/E. faecalis)*

Biofilm formation was measured in the following combinations:

- *S. boulardii* grown in MRS media with dextrose and inulin separately.
- *E. coli* grown in MRS media with dextrose and inulin separately.

- *E. faecalis* grown in MRS media with dextrose and inulin separately.
- *S. boulardii* and *E. coli* were mixed together and grown in MRS media having dextrose and inulin separately
- *S. boulardii* and *E. coli* were mixed together and grown in MRS media having dextrose and inulin separately

Cells were incubated at 30°C for 24 hrs and the planktonic bacteria were removed by shaking the plate out over a tray and washing. The wells were then stained with 1% crystal violet for 10 minutes followed by washing with water. The quantification of the biofilm production was carried out by dissolving the stained biofilm with 400µl of 30% acetic acid and measuring the absorbance at 630 nm using ELISA plate reader. Here n = 9.

	MRS (100µl)	INULIN (100µl)	M63 (200 µl)	*S. boulardii*	*E. coli*	*E. faecalis*
WELL1	■		■	■ 100µl)		
WELL2		■	■	■ 100µl)		
WELL3	■		■		■ (100µl)	
WELL4		■	■		■ (100µls)	
WELL5	■		■			■ (100µl)
WELL6		■	■			■ (100µl)
WELL7	■		■	■ (50µl)	■ (50µl)	
WELL8		■	■	■ (50µl)	■ (50µl)	
WELL9	■		■	■ (50µl)		■ (50µl)
WELL10		■	■	■ (50µl)		■ (50µl)

Statistical analysis: The results have been represented as the mean ± SD. *P* values less than 0.05 (two-tailed) were considered statistically significant.

2.4 RESULTS:

2.4.1 Relative growth of *S. boulardii* in presence of different prebiotics

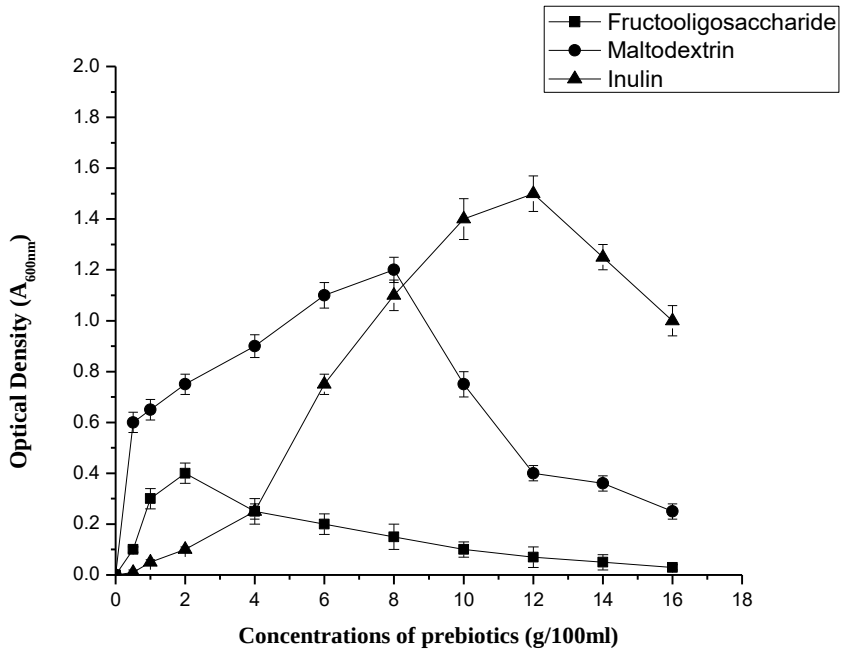


Figure 1: Effects of increasing concentration of prebiotics on the growth of *S.boulardii*

The relative growth of *S. boulardii* in presence of different concentrations of prebiotics such as fructooligosaccharide, maltodextrin and inulin has been shown in Figure 1. In this figure, X-axis represents the concentration of prebiotics in g/100ml and Y-axis represents OD at 600 nm. The result showed that fructooligosaccharide did not act as a prebiotic for *S. boulardii* as overall growth was very low in its presence. The maximum growth yield of *S. boulardii* was found at a dose of 0.02gm/ml in presence of fructooligosaccharide whereas it remained non-responsive at higher doses. The relative growth of *S. boulardii* was better in presence of maltodextrin than fructooligosaccharide and maximum growth was observed at a conc. of 0.08gms/ml. The OD and hence growth decreased with increase in concentration of maltodextrin indicated the reduction in growth rate. The growth of *S. boulardii* maximizes in presence of inulin and was found to be highest at 0.12gms/ml conc. The calculated t value far exceeds the tabulated t value with 5 degree of freedom at p=0.001.

2.4.2 in vivo monitoring of the effect of inulin on *S. boulardii* and other common gut bacteria

TABLE 1

	PREBIOTIC AND PROBIOTIC SUPPLEMENTS (SUPPLEMENTS GIVEN ONLY FOR 7 DAYS.)			
E. coli Count (CFU/ml)	Normal Control (No Treatment)	PREBIOTIC (Inulin) 1gm/kg body weight	PROBIOTIC (*S. boulardii*) 10^7 cells	PREBIOTIC + PROBIOTIC
After 7 days from start of the experiment	*Escherichia coli* ~10^9 *Enterococcus faecalis*~10^6 *Enterobacter aerogenes*~10^{10} *Staphylococcus sciuri*~10^{10} *Leuconostoc garvieae*~10^5 (~10^{10} CFU/ml)	*Escherichia coli*~10^4 *Enterococcus faecalis*~10^7 *Staphylococcus gallinarum*~ 10^4 (~10^7 CFU/ml)	*Escherichia coli*~10^5 + *S. boulardii* (~10^5 CFU/ml)	*Escherichia coli*~10^3 *Staphylococcus xylosus* ~10^4 *Leuconostoc Pseudomesenterica*~10^4 + *S. boulardii* (~10^4 CFU/ml)
After 14 days	Normal	Normal	Normal + *S. boulardii*	Normal + *S. boulardii*

Table 1 shows the effect of 7 days supplementation of inulin and *S. boulardii* on the growth of intestinal microbiota in mice as observed from stool samples. The normal count of microorganisms in the stool of a normal mouse was found to be ~10^{10} CFU/g with main organisms like *Escherichia coli, Enterococcus faecalis, Enterobacter aerogenes, Staphylococcus sciuri* etc. Interestingly, the count decreased to ~10^7 CFU/g in the mice received only inulin for 7 days with predominantly found *Escherichia coli, Enterococcus faecalis.* The number of microorganisms in stool sample further decreased to ~10^5 CFU/g in *S. boulardii* treated mice stool with predominant bacteria *Escherichia coli*. Pretreatment with both inulin and *S. boulardii* further lowered down the bacterial count in stool sample to ~10^4 CFU/g with bacteria like *Escherichia coli, Staphylococcus xylosus.* The calculated t value for *E. coli, Enterococcus sp.* and *Staphylococcus sp.* count in the stool sample of Swiss albino mice in control and prebiotic/probiotic treated sample far exceeds the tabulated t value with 5 degree of freedom at p=0.001. These observations led us to check if there was any association between *S. boulardii* and *Escherichia coli, Enterococcus sp.* and *Staphylococcus sp.* in vitro using phase contrast microscopy.

2.4.3 **in vitro experiments to check the interactions between *Saccharomyces boulardii* and other gut bacteria in presence of inulin using phase contrast microscope at 20X after 24 hours –**

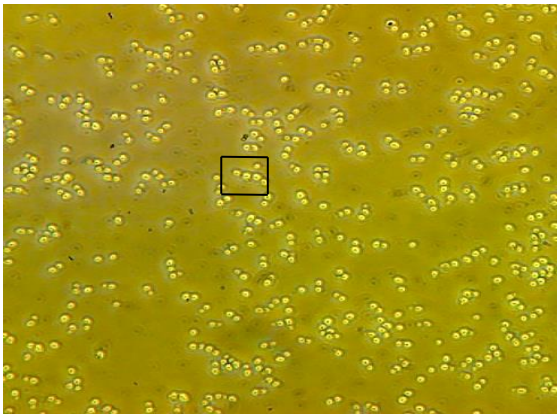
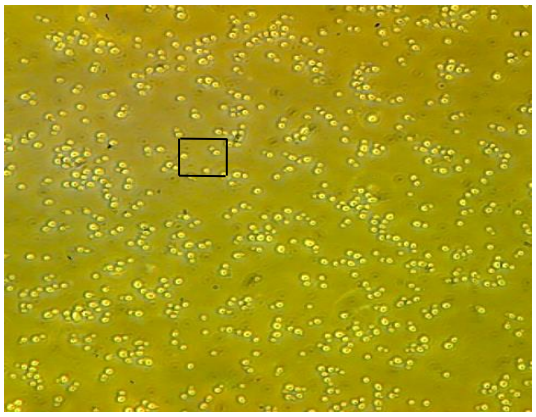


Figure 2.1A



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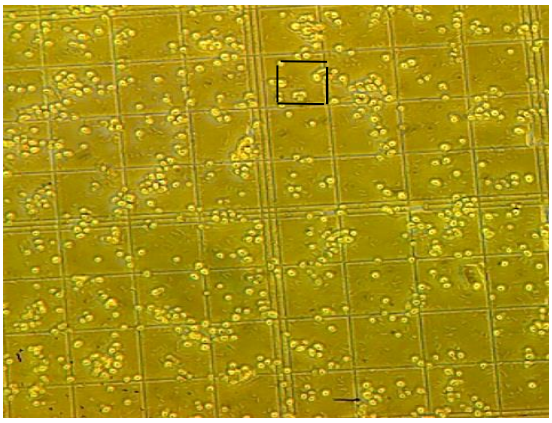

Figure 2.2

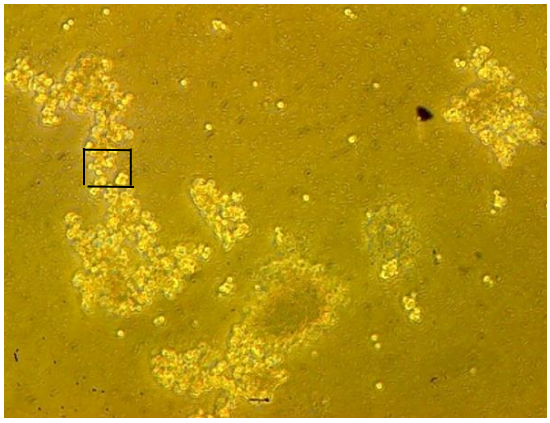

Figure 2.3

FIGURE 2: Image by phase contrast microscopy at 10X through phase plate of association between enteric pathogens and *S. boulardii* at various combinations of prebiotics after 24 hours of incubation at 30^{o}C. The square drawn in each photograph represents 0.25x0.25sq mm.

Figure 2.1A shows *S. boulardii* when grown at 30^{0}C in dextrose and Fig 2.1B shows *S. boulardii* when grown at 30^{0}C in inulin.

Fig 2.2 shows the image where *S. boulardii* was grown in dextrose and incubated with *E. coli* grown in nutrient broth and both the organisms were found to grow independently.

Fig 2.3 shows the image of *S. boulardii* grown in inulin and incubated with *E. coli* where clear association between these two was observed.

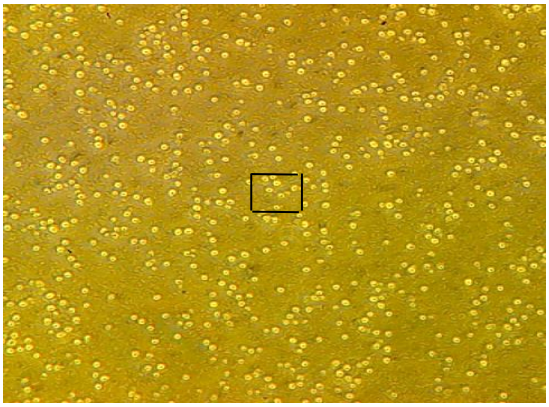

Figure 2.4

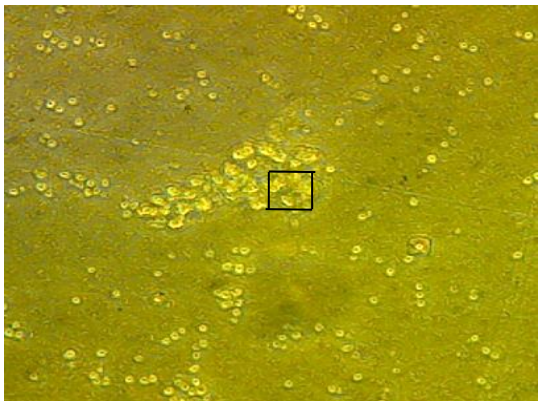

Figure 2.5

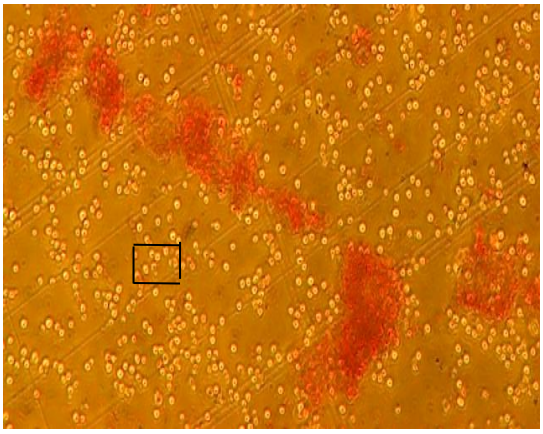

Figure 2.6

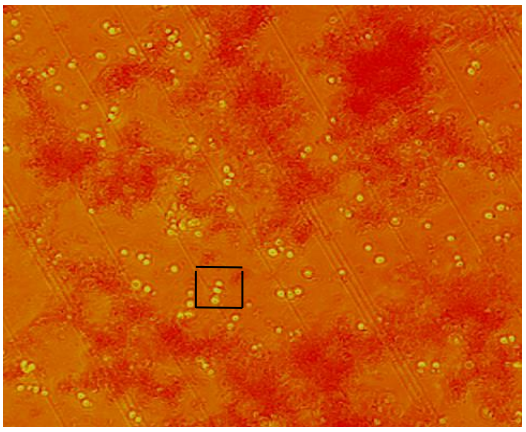

Figure 2.7

The image fig 2.4 shows that there was no association between *S. boulardii* and *E. faecalis* grown in dextrose. But close association was observed when *S. boulardii* was grown in inulin and incubated with *E. faecalis* and is shown in fig 2.5. The square drawn in each photograph represents 0.25x0.25sq mm.

Fig 2.6 and fig 2.7 shows that there was no association between *S. boulardii* and *Staphylococcus aureus* when grown in dextrose and inulin respectively.

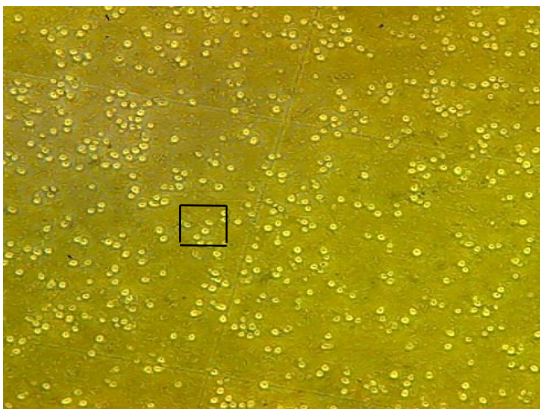

Figure 2.8

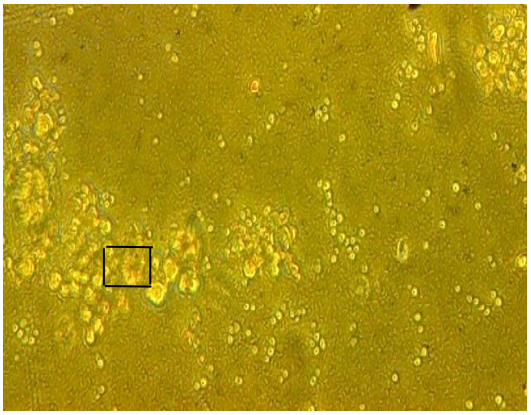

Figure 2.9

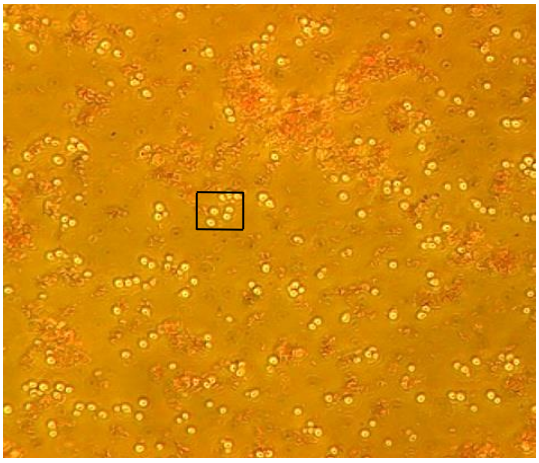

Figure 2.10

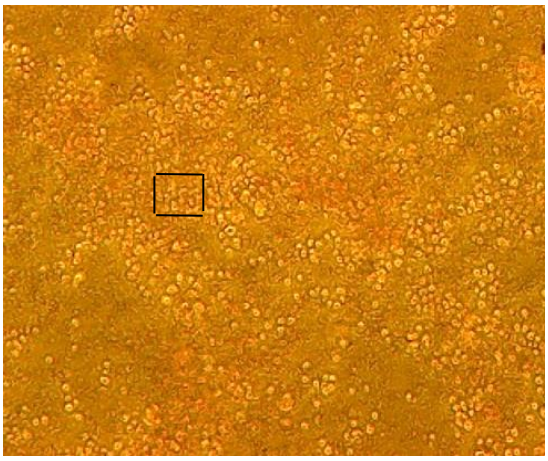

Figure 2.11

Fig 2.8 shows no aggregation among *S. boulardii, E. coli* and *E. faecalis* in dextrose whereas visible clumping and association among *E. coli, E. facealis* and *S. boulardii* was observed in the presence of inulin shown in fig 2.9. The square drawn in each photograph represents 0.25x0.25sq mm.

Fig 2.10 shows the image where *S. boulardii* grown in dextrose and incubated with *E. coli, S. aureus* and *E. faecalis*. Fig 2.11 shows *S. boulardii* grown in inulin and incubated with *E. coli, S. aureus* and *E. faecalis*.

Figure 2 shows the images captured by phase contrast microscope. It was observed that *S. boulardii* when grown at 30^0C in dextrose (fig 2.1A) or inulin (fig 2.1B) do not form any aggregate among themselves. Fig 2.2 shows the image where *S. boulardii* was grown in dextrose and incubated with *E. coli* grown in nutrient broth and both the organisms were found to grow independently. Fig 2.3 shows the image of *S. boulardii* grown in inulin and incubated with *E. coli* where clear associations between these two were observed. The image fig 2.4 shows that there was no association between *S. boulardii* and *E. faecalis* grown in dextrose. But close association was observed when *S. boulardii* was grown in inulin and incubated with *E. faecalis* and is shown in fig 2.5. Fig 2.6 and fig 2.7 shows that there was no association between *S. boulardii* and *S. aureus* when grown in dextrose and inulin respectively. Fig 2.8 shows no aggregation among *S. boulardii, E. coli* and *E. faecalis* in dextrose when mixed together whereas visible clumping and association among these three was observed in the presence of inulin as shown in fig 2.9. Similar experiment was performed with 3 strains of lactobacilli, *Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus rhamnosus*. No aggregation with *S. boulardii* was observed in case of these microorganisms when grown in inulin (results not shown). Finally, *Staphylococcus aureus, Escherichia coli* and *Enterococcus faecalis* was incubated with *Sacharomyces boulardii* in MRS and inulin, as expected in contrast to MRS they showed clumping nature in inulin media (Fig. 2.10 and 2.11). The amount of aggregate formation was checked by varying the concentration of inulin in the media and found that amount of aggregate is almost the same in all the concentrations of inulin. Hence, all the experiments were conducted at a concentration of 0.2gm/ml of inulin to check interaction. In all the experiments the aggregate formation started appearing from 3-4 hours but prominently observed only after 24 hours.

2.4.4 in vitro experiments to check the interactions between *Saccharomyces boulardii* and other gut bacteria in presence of maltodextrin and fructooligosaccharides using phase contrast microscope at 20X after 24 hours -

S. boulardii did not form aggregates with *Escherichia coli* or *Enterococcus faecalis* when grown in maltodextrin and fructooligosaccharides. The images were similar to the images where *S. boulardii* was grown in presence of dextrose (images not shown).

2.4.5 Time course study of aggregate formation

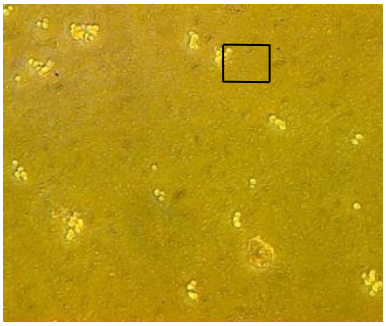

Figure 3.1

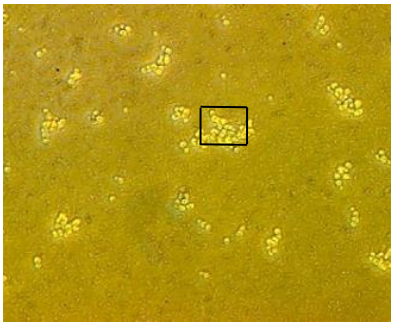

Figure 3.2

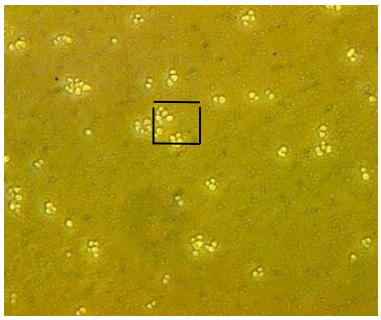

Figure 3.3

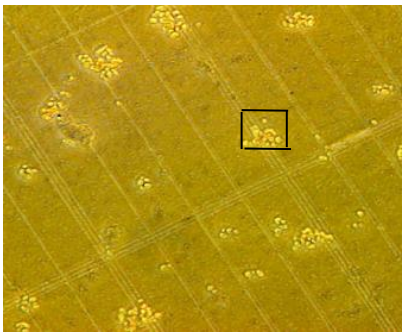

Figure 3.4

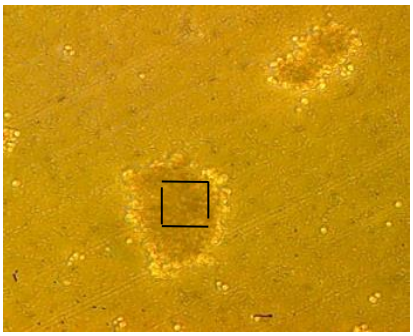

Figure 3.5

Fig 3.1 shows the association between *S. boulardii* and *E. coli* after 1 hr of incubation and Fig 3.2 shows association between *S. boulardii* and *E. coli* after 2 hrs of incubation. Fig 3.3 shows the association between *S. boulardii* and *E. coli* after 3 hrs of incubation. The square drawn in each photograph represents 0.25x0.25sq mm.

Fig 3.4 shows the association between *S. boulardii* and *E. coli* after 4 hrs of incubation and Fig 3.5 shows the aggregate formed after 24 hrs of incubation

Fig 3 shows the results of time dependent aggregation studies. The amount of aggregate was found to increase with time and maximum amount of aggregate was obtained around 24 hours.

2.4.6 Effect of heat treatment on aggregation between *S. boulardii* and common gut bacteria

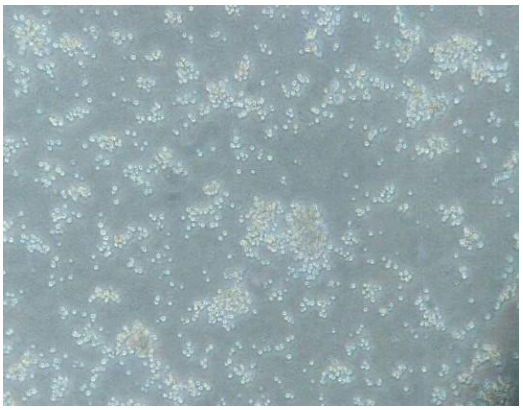

Figure 4.1

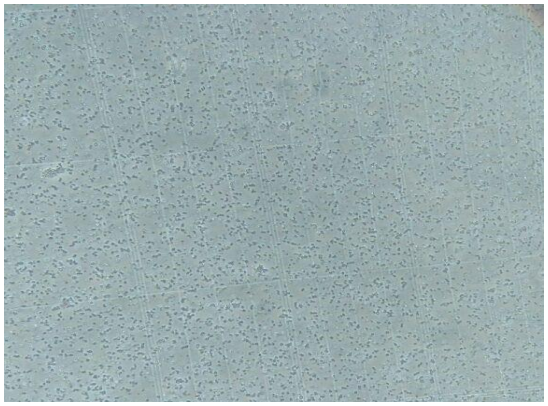

Figure 4.2

Fig 4.1 shows the interaction between *S. boulardii* grown in inulin and *E. coli*. Fig 4.2 shows no aggregation between heat-treated *S. boulardii* and *E. coli*. These results showed the necessity of intact cellular membrane component(s) in deciphering the property of aggregation.

2.4.7. Effect of cycloheximide on the aggregation property of *S. boulardii*

Figure 5.1

Figure 5.2

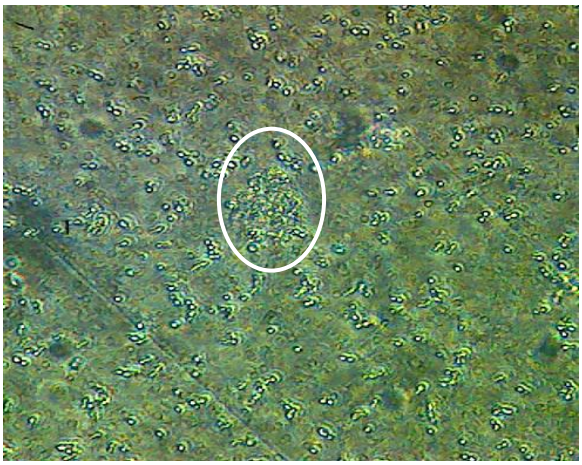

Figure 5.3

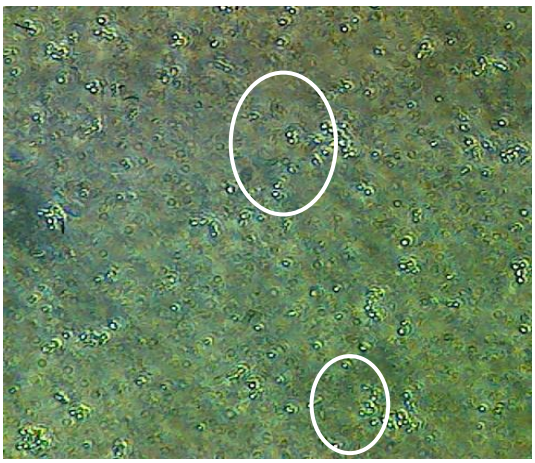

Figure 5.4

Figure 5 shows the effect of cycloheximide treatment on the aggregation property of *S. boulardii* grown in dextrose and inulin. The square drawn in each photograph represents 0.25x0.25sq mm.

Fig 5.1 shows the association between *S. boulardii* and *E. coli* in inulin after 4 hours and Fig 5.2 shows the association between cyclohexamide treated *S. boulardii* grown in inulin and incubated with *E. coli* for 4 hours

Fig 5.3 shows the association between *S. boulardii* and *E. coli* in inulin after 24 hours and Fig 5.4 shows association between cyclohexamide treated *S. boulardii* and *E. coli* in inulin after 24 hours

Association between *S. boulardii* and *E. coli*/*E. faecalis* shows that aggregate formation starts to appear after 4 hrs of incubation in inulin. The amount of aggregate formation was checked with cycloheximide treated *S. boulardii* and bacteria (Fig 5.2) and compared with control (Fig 5.1) after 4hrs of incubation. Results showed that there was no aggregation in the cycloheximide treated *S. boulardii* and *E. coli* /*E. faecalis* at 4hrs though little amount of visible aggregates appeared in the control. The amount of aggregate formation was also checked after 24 hours in cycloheximide untreated (Fig 5.3) and treated *S. boulardii* and found that aggregation slowly started to appear (Fig 5.4) with the elimination of the effect of cycloheximide from the cells. The result suggests that membrane proteins which might be the probable factors for aggregation did not appear on the yeast cell membrane until the effect of cycloheximide was nearly gone.

2.4.8 Result on biofilm assay

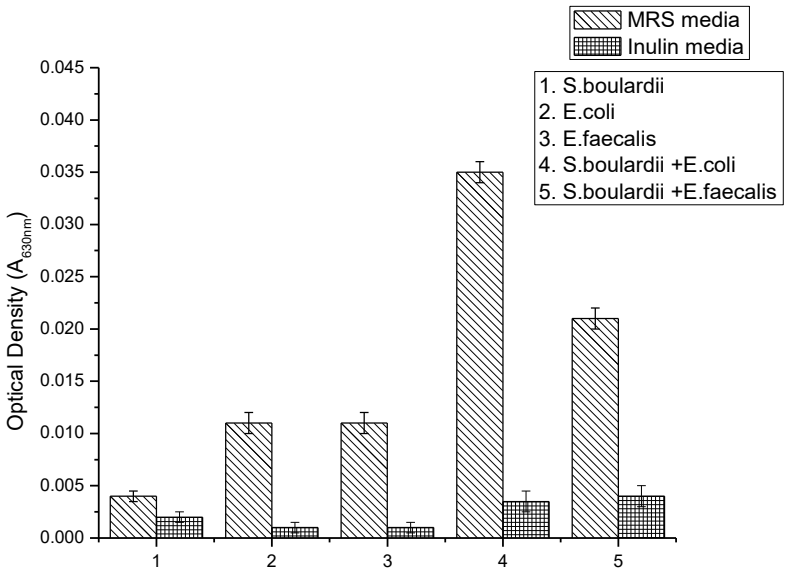

Figure 6: Biofilm assay

The results show Biofilm formation assays performed after addition of M63 Biofilm promoting media to either MRS or Inulin media.

Figure 6 shows the amount of biofilm formed in *S. boulardii*, *E. coli*, *E. faecalis* when grown individually in media having dextrose and inulin respectively and when *S. boulardii* was incubated together with *E. coli* or *E. faecalis* in presence of dextrose or inulin. The results show that the little amount of biofilm formed in presence of dextrose in case of *E. coli* or *E. faecalis* was reduced by inulin. The little amount of biofilm produced in dextrose by *E. coli* and *S. boulardii* was reduced in presence of inulin. The graph does not show any significant change in quantity of biofilm formed due to *S. boulardii* or inulin. Hence, biofilm synthesis is not the reason by which *E. coli* or *E. faecalis* remains attached to *S. boulardii*.

2.5 DISCUSSION:

The relative growth of *S. boulardii* was found to be significantly high in presence of inulin compared to maltodextrin and fructooligosaccharide. Maltodextrin acts as an effective prebiotic for *S. boulardii* at lower doses and the relative growth of *S. boulardii* decreases with the increase in maltodextrin concentration. Fructooligosaccharide was not very effective on the growth of *S. boulardii*. However, inulin cannot be called as the most efficient prebiotic for *S. boulardii* based on the growth parameter alone as inulin is effective only at high doses. Thus, instead of focusing on effective growth as our criteria we preferred observing the effect of inulin and *S. boulardii* on gut microflora.

The number of gut microorganisms was found to decrease gradually in the stool samples of mice when treated with *S. boulardii* and inulin separately or together compared to the number of microorganisms present in stool samples of normal mice. Corresponding *in vitro* assay on interactions between *S. boulardii* and common gut bacteria like *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus*, and different lactobacilli in presence of inulin showed that *S. boulardii* form aggregates with *Escherichia coli* and *Enterococcus faecalis* and the microorganisms remain alive in the clump. However, *S. boulardii* fails to form aggregate with these two bacteria when grown in presence of dextrose or other prebiotics like fructooligosachharides and maltodextrin. Perhaps the growth of *S. boulardii* and its association with *Escherichia coli* and *Enterococcus sp.* as observed in experiments could be the possible reason for decrease in number of *Escherichia coli* and *Enterococcus faecalis* in the gut surface and thus in the stool sample of mice after 7 days of treatment with *S. boulardii* and *S. boulardii* along with inulin. Similar evidence has been found from other studies that bacteria which do not attach to the surface of the gut are removed by the mucus (Kalliomaki et al., 2008). Thus, it can be assumed that the association which was found in the *in vitro* assay must have been formed within the mice gut that resulted in its excess removal in the same way. The reduction in number of microorganisms in the stool samples of mice received only inulin could be due to the overgrowth of bifidobacteria as well as destructive property of inulin against biofilm formation by various infectious agents including *S. aureus* as reported in the literature (Sampo et al., 2007).

Besides this, *S. boulardii* covers large area compared to those of bacteria hence it gives good habitat competition to other pathogens, that resulted in pathogen count reduction in mice feed with probiotic alone. *in vitro* and *in vivo* experiments demonstrated that inulin irrespective of its concentration can enhance the probiotic properties of *S. boulardii* specifically for some bacteria and simultaneously it also facilitates modulation of the balance in the intestinal microflora in a beneficial way. Although it aggregates with *E. coli* and *E. faecalis*, it did not form any attachment among themselves as well as with

other probiotic bacteria, thus making it very specific regarding expression of any protein that may be involved in this clumping.

The amount of aggregate formation was independent of the concentrations of inulin in the medium. The size of the aggregate increased with time and maximum amount of aggregate was obtained around 24 hours. Heat killing of *S. boulardii* completely abolished the aggregate forming capability and it can be assumed that intact cell wall is required for deciphering the property of aggregation. Results on biofilm formation showed that the little amount of biofilm synthesis took place in MRS by *S. boulardii* and *E. coli* was inhibited in presence of inulin. Hence, biofilm synthesis is not the reason by which *E. coli* or *E. faecalis* remains attached to *S. boulardii*. Certain surface molecules like proteins which are expressed in presence of inulin on the cell surface of *S. boulardii* might be responsible for binding to certain factors on the surface of *E. coli* or *E. faecalis*. Hence, aggregation was studied after treating *S. boulardii* with a potent translation inhibitor, cycloheximide. Results showed that *S. boulardii* treated with cycloheximide showed no aggregation with *E. coli* and *E. faecalis* after the withdrawal of cycloheximide for 4-5hrs. Aggregates started to appear again after the effect of cycloheximide was gone completely from *S. boulardii* and the amount of aggregation at 24hrs was comparable with untreated *S. boulardii*. Thus possibly *S. boulardii* expresses specific proteins on its surface when grown in inulin and those proteins are responsible for binding to *E. coli* and *E. faecalis*.

Thus, the observed *in vivo* results of decrease in number of microorganisms might be because of the aggregates formed between bacteria present in gut and *S. boulardii* in presence of inulin and their subsequent elimination through mucus. *S. boulardii* do not generally possess any surface feature to attach with bacteria when grown in dextrose but it probably shows expression of certain factors in presence of inulin, it could be surface protein as heat killed cells were unable to show any association and cycloheximide treatment inhibited the aggregation. Thus, synbiosis can boost and manipulate the gut flora better than the sole use of probiotics and might have more clinical implications.

Research in this thesis is not sufficient to comment on the nature of these factors which are making *S. boulardii* more specific about interaction and forming clumps with some bacteria, but it gives an idea that prebiotics not only serves as carbon source but can interfere with protein expression too.

2.6 CONCLUSION:

- *S. boulardii* grows better in presence of inulin compared to other prebiotics like maltodextrin and fructooligosaccharides.
- *in vivo* treatment with *S. boulardii* and inulin reduces the number of microbes in the feces of mice treated with inulin, *S. boulardii* together or separately.
- *S. boulardii* grown in inulin forms aggregates with *E. coli* / *E. faecalis* in vitro but not with *S. aureus* or lactobacilli.
- Aggregate formation does not take place in presence of prebiotics like maltodextrin or fructooligosaccharide.
- The amount of aggregate increases with time and maximum amount was observed around 24 hrs.
- The amount of aggregate did not increase with increase in concentration of inulin concentration.
- Live *S. boulardii* is required for the interaction to take place with *E. coli* and *E. faecalis.*
- Treatment of *S. boulardii* with cycloheximide reduces its aggregate formation capability with *E. coli* and *E. faecalis* suggesting a role of membrane proteins in the association.

2.7 Reference

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CHAPTER 3

Role of prebiotics on the growth and antibiotic sensitivity of probiotic lactobacilli

3.1 INTRODUCTION

The intestine of a human is inhabited by a diverse microbial community with specialized function and important physiological effects. The dynamic cross-talks between the host and the microbiota have a beneficial effect on the host homeostasis in health and disease, including activation of the immune system. In 21st century, sufferings from infectious diseases has lead us to use antibiotics in our day to day life for temporary relief but high doses of antibiotics are capable of killing approximately 95% of all microbes that have profound effect on the chemical composition of the gut. It is evident that over 87% of all molecules detected in the gut had their levels significantly altered by antibiotic treatment in a mouse intestine (Antunes et al, 2011). At the same time, many antibiotics have side effects on our physiology. Thus, antimicrobial alteration of the microbiota should be replaced by alternatives like administration of probiotics, prebiotics and synbiotics (Chen et al, 2014). Synbiotics, the combination of probiotics and prebiotics have become the subject of increasing interest in manipulating the enteric microbiome, trillions of microbes that inhabit the human digestive tract. Probiotics exert beneficial health effects in the host by limiting colonization and proliferation of gut pathogens such as *Salmonella* sp., *E. coli*, *Enterococcus* sp. etc. (Patel and Goyal et al, 2012). It has been found that antibiotic induced gut microbiota dysbiosis promotes tumor initiation, while supplementation of LPS has shown to suppress it (Aguiler et al, 2015). Administration of *Lactobacillus plantarum* LS/07 alone or with inulin for a long period has been found to be effective against breast cancer (Kassayová M et al, 2014). Although substantive amount of research is going on the interactions between the microbiota, host and the prebiotics, there are indications of the major beneficial effects of pre and probiotic if used judiciously.

In this study, effects of three prebiotics viz fructooligosaccharide (FOS), maltodextrin and inulin have been tested on the growth and antibiotic sensitivity of three probiotic lactobacilli, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus casei*.

3.2 OBJECTIVES

- Comparative analysis of the effects of prebiotics, fructo-oligosaccharide, inulin and maltodextrin on the growth of *Lactobacillus spp.* (*Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus casei*)
- Comparison of the sensitivity of the probiotics, *Lactobacillus* spp, in the presence and absence of prebiotics against commonly used antibiotics like ciprofloxacin, azithromycin, ampicilin.
- Probable causes/mechanisms behind high susceptibility of lactobacilli towards certain antibiotics in presence of specific prebiotic.

3.3 Materials and methods

3.3.1 Strains of Probiotics and growth condition

Lactobacillus casei (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462) were collected from MTCC (The Microbial Type Culture Collection), India.

Lactobacillus rhamnosus 5300 and *Lactobacillus plantarum* 2083 were collected from National collection of industrial microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune.

Lactobacillus plantarum and *Lactobacillus casei* were maintained by routine sub-culturing in the Man-Rogosa-Sharpe (MRS, HiMedia, India) broth using 1% (v/v) inoculums from an overnight culture and incubated in BOD incubator at 37^0C.

3.3.2 In vitro monitoring of the effect of maltodextrin on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462)

All the lactobacilli were grown in MRS (Man-Rogosa-Sharpe) broth, which specifically supports the growth of *Lactobacillus* spp. The carbohydrate source dextrose present in MRS broth was replaced by maltodextrin. 200µl or 1% (v/v) of log-phase culture of three lactobacillus strains were introduced separately into the nephalometric flasks containing MRS broth with dextrose (control) and different concentrations of maltodextrin (1%, 2%, 5% and 10% w/v). Flasks were incubated at 37^0C in shaker. To test the prebiotic efficiency of maltodextrin, growth was checked in each flask by measuring corresponding O.D. values at 600nm with a UV-VIS spectrophotometer, at an interval of 1hour upto 24hrs.

3.3.3 In vitro monitoring of the effect of Inulin on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462)

All the lactobacilli were grown in MRS (Man-Rogosa-Sharpe) broth, the commonly used growth medium for *Lactobacillus* spp. The carbohydrate source dextrose present in MRS broth was replaced by inulin. 200µl or 1% (v/v) of log phase culture of three lactobacillus strains were introduced separately into the nephalometric flasks containing MRS broth with dextrose (control) and MRS broth with different concentrations of inulin (1%, 2%, 5% and 10% w/v). Flasks were incubated at 37^0C in shaker. To test the effect of Inulin on the growth of lactobacillii, growth was checked in each flask by measuring corresponding O.D. values at 600nm with a UV-VIS spectrophotometer after 24hrs.

3.3.4 In vitro monitoring of the effect of Fructooligosaccharides on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462)

All the lactobacilli were grown in MRS (Man-Rogosa-Sharpe) broth, which specifically supports the growth of Lactobacillus spp. The carbohydrate source dextrose present in MRS broth was replaced by fructooligosaccharide. 200µl or 1%,(v/v) of log-phase culture of three lactobacillus strains were introduced separately into the nephalometric flasks containing MRS broth with dextrose (control) and MRS broth with different concentrations of fructooligosaccharide (1%, 2%, 5% and 10% w/v). Flasks were incubated at 37^0C in shaker. To test the prebiotic efficiency of Fructo oligosaccharide, growth was checked in each flask by measuring corresponding O.D. values at 600nm with a UV-VIS spectrophotometer after 24hrs.

The relative growth of organisms on a prebiotic substrate = (A/B) × 100% where, A = Mean OD_{600} value of a strain on prebiotic substrate and B = mean OD_{600} value of the same strain grown on dextrose. The results would reflect the growth of a specific probiotic strains on a specific prebiotic substrate relative to its growth on a dextrose substrate which was fixed at 100%.

3.3.5 Effect of prebiotics on the antibiotic sensitivity of probiotics

Minimum inhibitory concentration measurement test is performed in presence of prebiotics using EZY MIC^{TM} strips. Minimum inhibitory concentrations (MIC) of the antibiotics were determined for the organisms in the presence and absence of prebiotics over control condition. Results were determined by measuring the formation of inhibition zones on the bacterial lawn. The following antibiotic strips of specific concentrations were used for the experiments.

Ampicillin- 0.016 to 256 μg/ml, Azithromycin 0.016 to 256 μg/ml and Ciprofloxacin 0.002 to 32 μg/ml E-tests were performed with azithromycin and ciprofloxacin to find out the effects of prebiotic on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462). The cells were grown in MRS agar (with dextrose) and modified MRS agar where dextrose had been replaced by specific prebiotics. 250 μl of log-phase culture was spread evenly onto MRS agar and modified agar. Antibiotic strips were then placed in the middle of the petri dishes to find out the MIC of the bacteria in presence and absence of prebiotic. The petri dishes were incubated overnight at 37^0C and zone of inhibition was observed.

3.3.5.1. Study of the effect of antibiotic on the viability of *L. casei* in presence of the prebiotic maltodextrin by MTT assay

L. casei (MTCC NO.-1423) strains were grown in presence and absence of maltodextrin in MRS broth for 3 hours to attain the log phase. Azithromycin at a concentration of 250μg/ml (highest concentration present in the MIC strip) was added in all the concerned tubes except the control ones which had only cell cultures. All the tubes were incubated for 1 hour at 37^0C and then 200μl of MTT reagent [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added to 500 μl of cell suspensions taken from each tube and incubated for 1 hour at 37^0C in dark before measuring O.D. at 590nm (Chakrabarti et al, 2000).

3.3.5.2. To check the pH in *L. casei* (MTCC NO.-1423) growth media:

The pH of both the culture media i.e. with dextrose and with maltodextrin was measured at regular interval and after 24 hours of growth.

3.3.5.3. To check the effect of low pH on the viability of *L. casei* (MTCC NO.-1423)

Antibiotic sensitivity assay was conducted in dextrose media with low pH.

3.3.5.4. To check the effect of maltodextrin on cellular morphology using scanning electron microscopy (SEM)

The morphology of *L. casei* (MTCC NO.-1423) grown in dextrose and maltodextrin was checked using scanning electron microscopy analysis. Overnight grown *L. casei* cells were centrifuged and the pellet was resuspended in phosphate buffer saline (PBS). 50µl of resuspended cells were placed on the grease free coverslip and air dried. The cells were fixed at room temperature for 2 hours with 2.5% glutaraldehyde. The coverslips were rinsed with PBS for 3 times. The cells were dehydrated using increasing concentrations of ethanol sequentially. First, the cover slips were dipped in 50% ethanol for 10 minutes, then in 70% ethanol for another 10 minutes, next in 80% ethanol for 10 minutes followed by 95% ethanol with 2 changes within 10 minutes and finally into 100% ethanol with 3 changes within 15 minutes (http://www.cmif.osu.edu/Methods-SEM).

3.3.5.5. To check the effect of maltodextrin on the membrane permeability of lactobacilli using fluoroscence microscopy

The permeability of the cell membranes of the strains *Lactobacillus rhamnosus* 5300 and *Lactobacillus plantarum* 2083 grown in presence of maltodextrin was checked using fluorescence microscopy. Both the strains were grown in MRS broth containing dextrose as carbon source at 37^0C for 24hrs. Then the overnight grown cultures were inoculated separately in MRS broth containing dextrose and maltodextrin as carbon source. After 24hrs of incubation, the samples were centrifuged at 7000 rpm for 10mins. The pellets were resuspended in 1 ml 0.85% NaCl, vortexed for 10 mins and microfuged again for 5 mins. These washing steps were performed twice and the pellets were resuspended in 1ml 0.85% NaCl and stained with 3 µl of 1:1mixture of 20mM propidium iodide and 0.01% (w/v) acridine orange. Stained samples were vortexed and incubated in the dark at room temperature for 15 mins. Dyes were removed by microfuging and resuspending in 1ml of 0.85% NaCl. Each sample was diluted 100 folds in sterile saline. A wet mount was prepared from this dilution with 50% glycerol and observed under fluorescence microscope set with 490 nm excitation filter (Michael et al, 2014).

Statistical analysis

The results have been represented as the mean ± SD. *P* values less than 0.05 (two-tailed) were considered statistically significant.

4.4 RESULT

4.4.1 Effect of maltodextrin on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462)

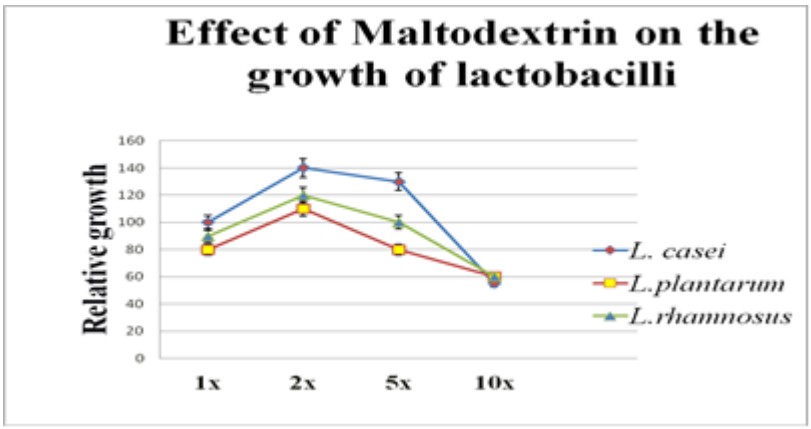


Figure 1: The effect of the prebiotic maltodextrin on the three probiotic strains are shown in figure 1. FOS and maltodextrin act as good prebiotics for *L. casei*.

Relative growth has been calculated as = (A/B) × 100% where, A = Mean OD_{620} value of a strain on prebiotic substrate and B = mean OD_{620} value of the same strain grown on dextrose.

Relative growth of all the strains was found to be maximum at 2 x concentration of maltodextrin. Relative growth decreased with increase in maltodextrin concentration which is probably due to the increase in osmolarity of the growth media.

3.4.2 Effect of inulin on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462)

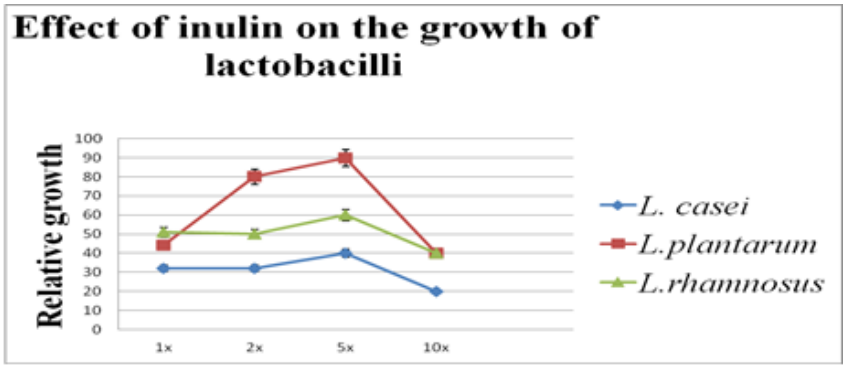


Figure 2 shows the effect of inulin on the growth of three strains of lactobacilli (x represents 1% of prebiotic). Inulin did not act as a prebiotic for *L. casei* and it had very little prebiotic effect on *L. rhamnosus*. The growth rate of *L. plantarum* increased with increasing concentration of inulin and maximum growth was observed at a concentration of 5gms/100ml.

3.4.3 Effect of fructooligosaccharide on the growth of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462)

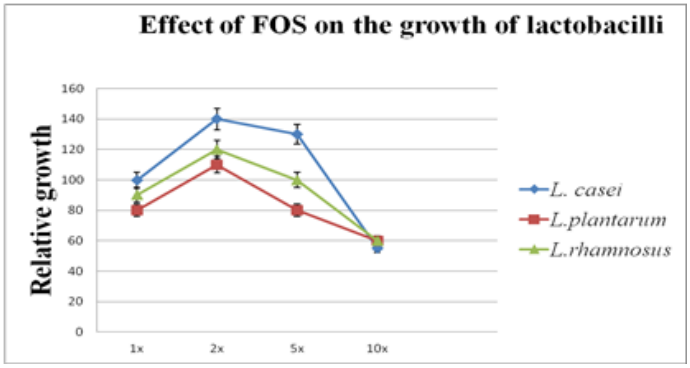
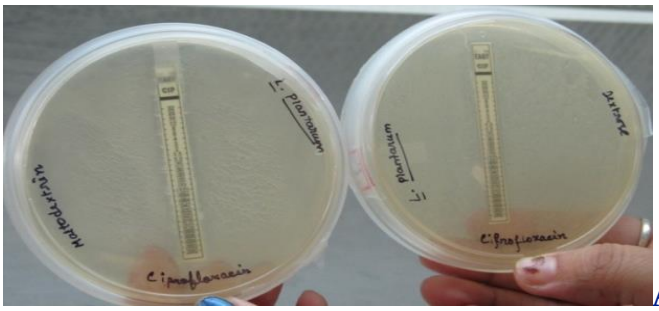


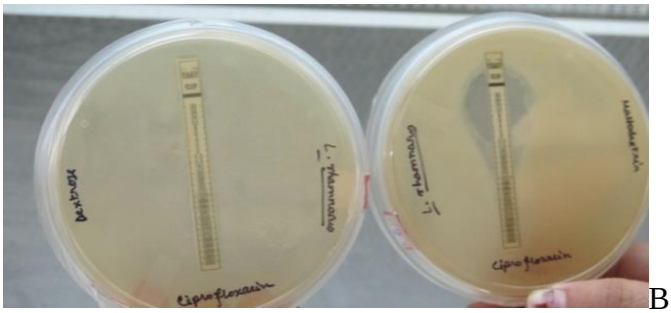
Figure 3 shows the effect of the prebiotic fructooligosaccharide (FOS) on the growth of three Lactobacillus strains. All the strains were found to grow better at lower concentration of fructooligosaccharide i.e. 1x and 2x (1 and 2 gm/100 ml).The growth response of *L. casei* was the best among the three strains in response to FOS and maximum relative growth was observed at a concentration of 2gms/100ml.

3.4.4. Sensitivity of the microorganisms towards antibiotics in presence of different prebiotics (5x) over dextrose

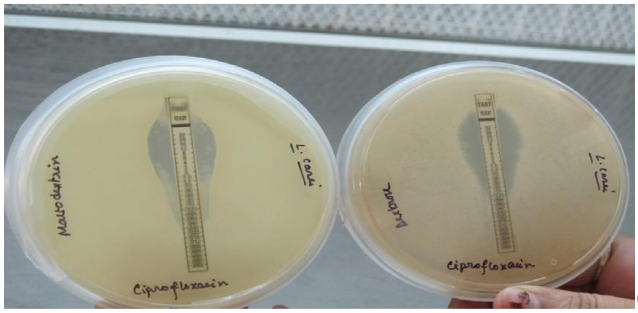
3.4.4.1 MIC value of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462) with respect to antibiotic Ciprofloxacin in presence (right) & absence of the prebiotic (left) maltodextrin



A



B



C

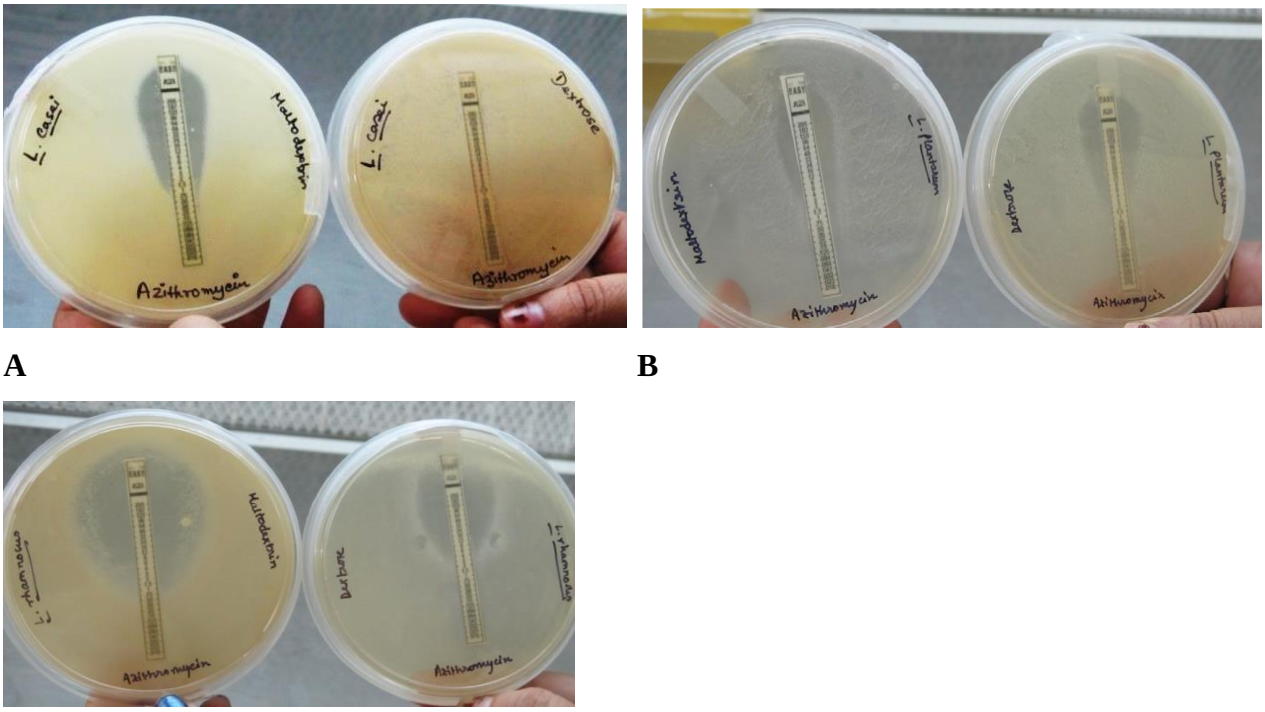
Figure 4 shows the result of E-test performed with ciprofloxacin MIC (conc. range- 0.002 to 32µg/ml) strips.

L. plantarum did not show any difference in its ciprofloxacin sensitivity in presence and absence of maltodextrin (Fig 4A). The organism was resistant to the dose of ciprofloxacin used for this study.

The presence of maltodextrin in media showed to reduce the MIC value of the antibiotic ciprofloxacin for *L. rhamnosus.* The MIC value was found to be 0.032 µg/ml in presence of maltodextrin whereas no inhibition was found in presence of dextrose at the highest concentration (32 µg/ml) used for this study (Fig 4B).

L. casei showed slightly increased ciprofloxacin sensitivity in the presence of maltodextrin (Fig 4C). The MIC value was 0.125µg/ml when grown in dextrose but the value decreased to 0.032µg/ml in presence of maltodextrin.

3.4.4.2 MIC value of *Lactobacillus casei* (MTCC NO.-1423), *Lactobacillus rhamnosus* (MTCC NO.1408), *Lactobacillus plantarum* (MTCC NO. 4462) with respect to antibiotic Azithromycin in presence & absence of the prebiotic maltodextrin



A **B**

C

Figure 5- Result of E-test with Azithromycin (Conc. Range-0.016 to 250µg/ml) in presence and absence of maltodextrin.

Figure 5A shows the result of E-test performed with Azithromycin strips on *L. casei* in presence of maltodextrin and dextrose. There was no inhibition zone in control plate (with dextrose) but a clear zone of inhibition was observed in the plate containing prebiotic (maltodextrin). *L. casei* was resistant to the highest dose of azithromycin used in the study in the presence of dextrose but showed increased sensitivity in presence of maltodextrin and the MIC value was as low as 0.50µg/ml.

L. plantarum showed no change in its azithromycin sensitivity in the presence of maltodextrin compared to dextrose (Fig 5B). The MIC value of azithromycin for *L. plantarum* was found to be 1.5 µg/ml in both dextrose and maltodextrin.

Figure 5C shows the effect of maltodextrin on the azithromycin sensitivity of *L. rhamnosus*. This strain becomes more sensitive towards azithromycin in presence of maltodextrin compared to dextrose.

3.4.5 Antibiotic sensitivity chart of LAB in presence of prebiotics

	Streptomycin (MIC value in μg/ml)	**Ampicillin (MIC value in μg/ml)**	**Azithromycin (MIC value in μg/ml)**	**Ciprofloxacin (MIC value in μg/ml)**
Lactobacillus casei				
MRS media	**8±0.5**	**-**	**-**	**-**
Inulin media	**-**	**-**	**-**	**-**
Maltodextrin media	**5±0.1**	**-**	**-**	**-**
Lactobacillus plantarum				
MRS media		**3±0.2**	**2±0.1**	**32±2**
Inulin media	**No Inhibition zone**	**No Inhibition zone**	**3±0.2**	**No Inhibition**
Maltodextrin media	**64±3**	**-**	**-**	**8±0.6**
Lactobacillus rhamnosus				
MRS media	**192±10**	**-**	**-**	**16±1**
Inulin media	**No Inhibition zone**	**3±0.2**	**8±0.2**	**No Inhibition zone**
Maltodextrin media	**-**	**-**	**-**	**16±1.5**

Values equal to 2 μg/ml or more have been considered

3.4.6. MTT assay of *L. casei* (MTCC NO.-1423) cells grown in presence and absence of maltodextrin and subjected to antibiotic (Azithromycin).

Figure 6 compares the viability of *L. casei* in presence of dextrose and maltodexrin and effects of Azithromycin on it.

MTT assay is performed to assess the metabolic activity of cells to measure cytotoxicityi.e. loss of viable cells. The enzymes NAD(P)H dependent cellular oxidoreductase are capable of reducing the tetrazolium dye MTT or 3-(4,5 dimethyl thiazal -2 yl) 2,5 diphenyl tetrazolium bromide which readily penetrates into the insoluble cells to form formazan.Hence more formazan production is proportional to the viability or metabolic activity of a cell. The viability of *L. casei* growing in dextrose is considered 100% from the result of MTT assay. The viability of other cells was calculated accordingly. The viability of *L. casei* decreased significantly to 72% when grown in maltodexrin. Application of antibiotics on *L. casei* grown in dextrose and maltodexrin reduced the cell viability to 85% and 65% respectively.

3.4.7. Change in pH of *L. casei* (MTCC No. 1423) culture media with dextrose and maltodextrin with time

MEDIA WITH	TIME (in Hrs)				
	3 Hrs	4 Hrs	5 Hrs	6 Hrs	24 Hrs
Dextrose (pH)	6.61± 0.0058	6.56± 0.0041	6.46± 0.013	6.39± 0.0041	4.90± 0.009
Maltodextrin (pH)	6.65± 0.0041	6.59± 0.007	6.53± 0.0041	6.44± 0.007	3.50± 0.008

TABLE 2. Time course of change in pH of *L. casei* (MTCC NO.-1423) culture media with dextrose and maltodextrin

Table-1 shows the change of pH in both the media (with dextrose and maltodextrin) initially after 3hours and at every 1 hour of interval and after 24 hours. When compared, the pH change in initial hours of growth in both the media appeared to be close to each other. The last reading of pH of both the media corresponds to the pH measured after 24 hours of growth. A drop in pH was observed in maltodextrin after 24 hours compared to dextrose which is significantly different ($P \leq 0.001$) from pH of the control (dextrose). Results are expressed as mean ± standard error of means, n = 6.

Results showed no change in *L. casei* cellular morphology when the PH of the media containing dextrose was lowered down to that of maltodexrin. Hence, change in PH or lowering of PH is not the reason for decreased cellular metabolic activity or less cell viability.

3.4.8. Determination of the morphological changes in *L. casei* (MTCC NO.-1423) in presence of maltodextrin and azithromycin

Figure 7 depicts the SEM photographs of the *L. casei* cells grown in dextrose and maltodextrin and subject to azithromycin.

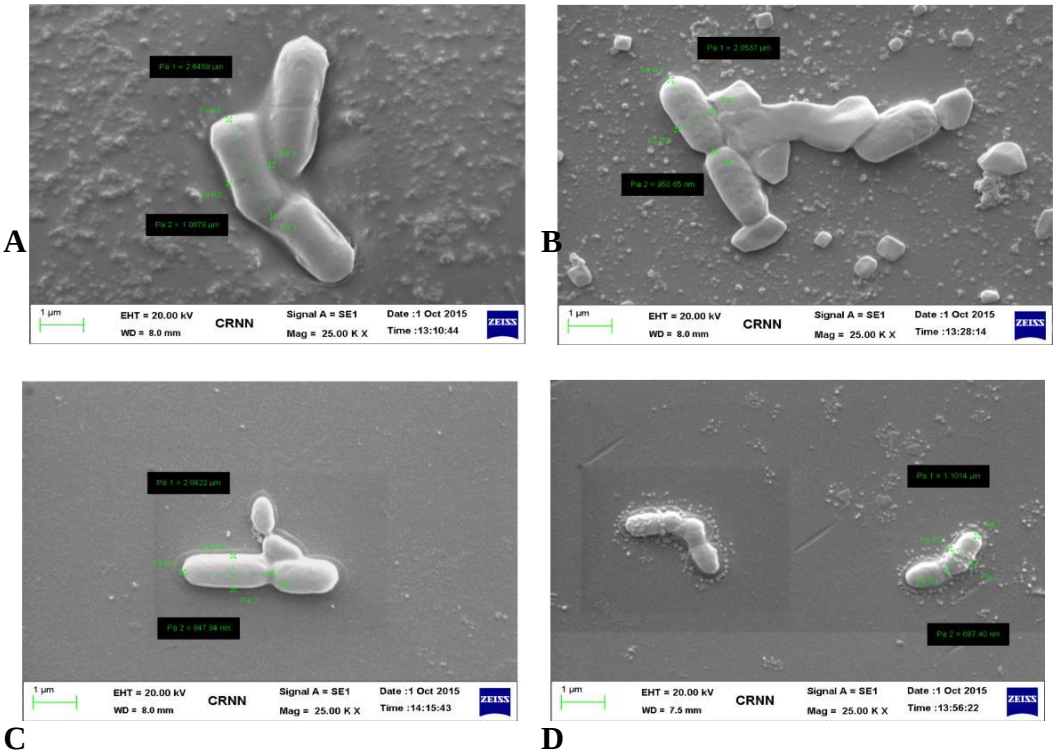
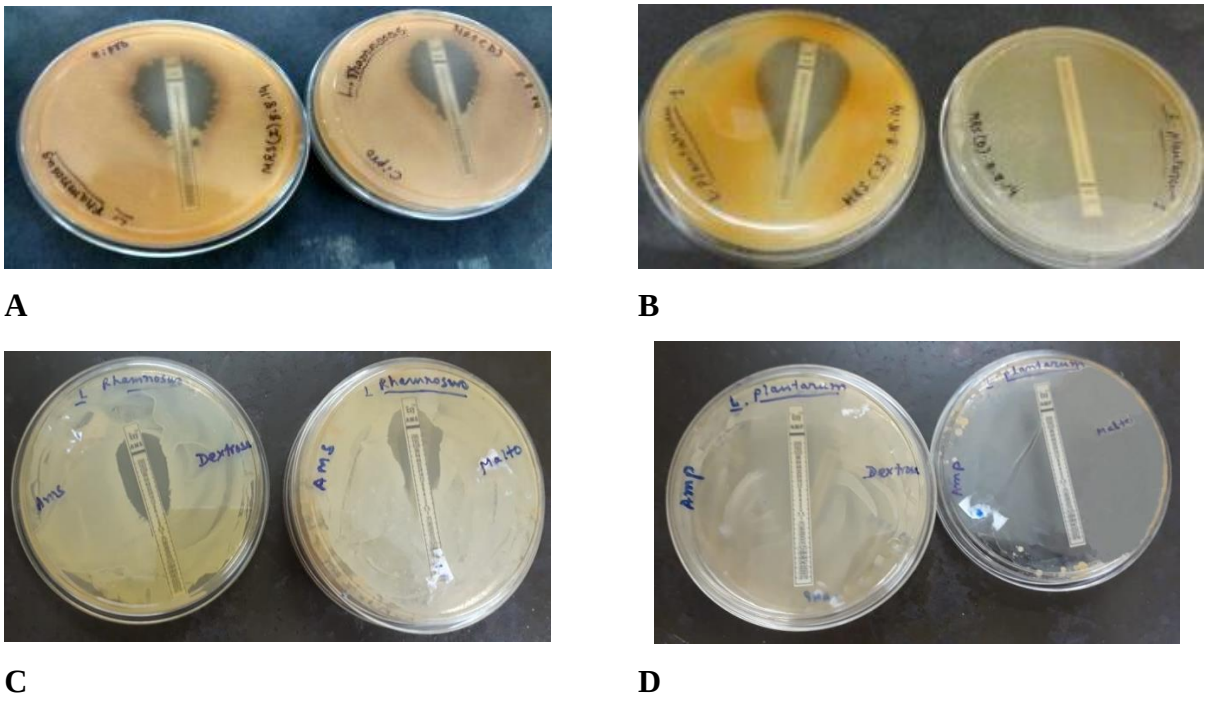


Figure 7- SEM photographs of the *L. casei* cells grown in dextrose and maltodextrin and subject to antibiotics.

L. casei (Bar- 1µm) grown in dextrose (B) grown in dextrose and exposed to Azithromycin (C) grown in maltodextrin (D) grown in maltodextrin and exposed to Azithromycin (D)

The length and breadth of *L. casei* grown in dextrose was found to be 2.5±0.0049 µm and 1.15± 0.0069 µm respectively. Both the dimensions reduced when exposed to Azithromycin and became 2.21±0.05 µm and 0.942±0.01 µm. The interesting observation was that the cells became much shorter, almost half, when grown in presence of maltodextrin, 1.2±0.110 µm and 0.640±0.033 µm respectively. The cells further reduced to 1.2±0.05 µm and 0.60±0.040 µm when grown in maltodextrin and exposed to Azithromycin.

3.4.9 MIC value of *Lactobacillus rhamnosus* 5300 and *Lactobacillus plantarum* 2083 with respect to antibiotic ciprofloxacin and ampicillin in presence (right) & absence of the prebiotic (left) inulin [A and B] and Maltodextrin [C and D]



A **B**

C **D**

Figure 8

The sensitivity of *L. rhamnosus* towards ciprofloxacin was found to be the same both in presence of dextrose and inulin (Fig 8A). The sensitivity of *L. plantarum* increased towards ciprofloxacin in the presence of inulin. *L. plantarum* which was resistant to the highest dose of ciprofloxacin (32µg/ml) used in this study when grown in dextrose showed increased sensitivity when grown in maltodextrin (Fig 8B). Similar observation was obtained when *L. rhamnosus* and *L. plantarum* was grown in dextrose and maltodextrin and subject to ampicillin. *L. plantarum* showed high sensitivity in presence of ampicillin in maltodextrin. The extremely low MIC of the antibiotics for *L. plantarum* is an indication of possible membrane damage in this bacterium. Hence to check the membrane integrity, MTT assay was performed with *L. plantarum* 2083 and *L. rhamnosus* 5300 after growing in dextrose and maltodextrin.

3.4.10 Cell viability of *L. rhamnosus* 5300 grown in dextrose and maltodextrin and exposed to antibiotic (Ampicilin)

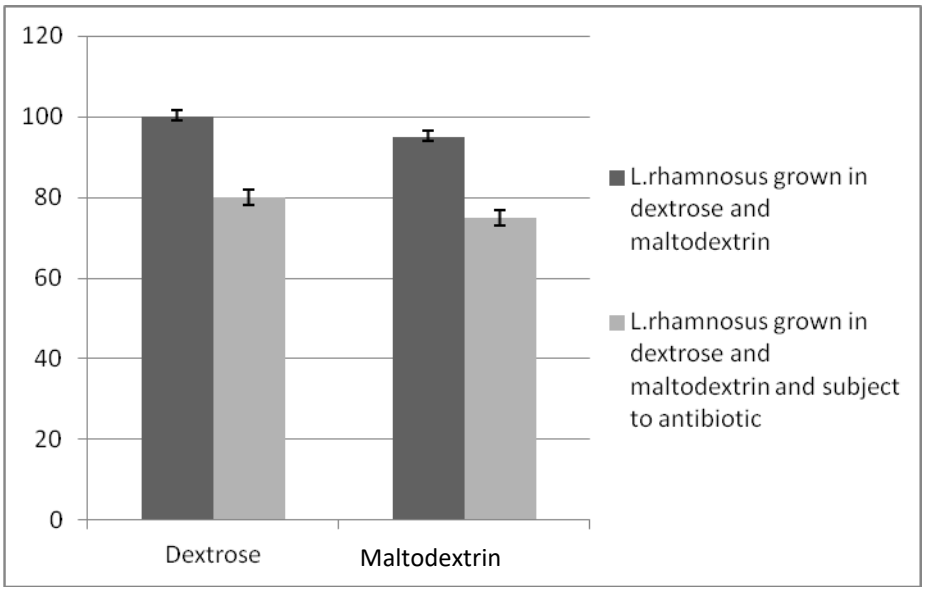


Figure 9 shows the change in cell viability of *L. rhamnosus* in response to the antibiotic Ampicilin in presence of dextrose and maltodextrin**.**

The viability of *L. rhamnosus* was considered as 100% when grown in dextrose. Viability of other cells was calculated accordingly from the MTT assay result. Viability of *L. rhamnosus* was reduced slightly to 95% in presence of maltodexrin. Treatment of the cells with ampicilin reduced the viability of cells in dextrose and maltodexrin to 80% and 75% respectively. Hence, it can be said that maltodextrin has no profound effect on the *L. rhamnosus* cell viability which is also supported by the antibiotic sensitivity of the cell both in the presence of dextrose and maltodextrin.

3.4.11 Cell viability of *L. plantarum* 2083 grown in dextrose and maltodextrin and subjected to antibiotic

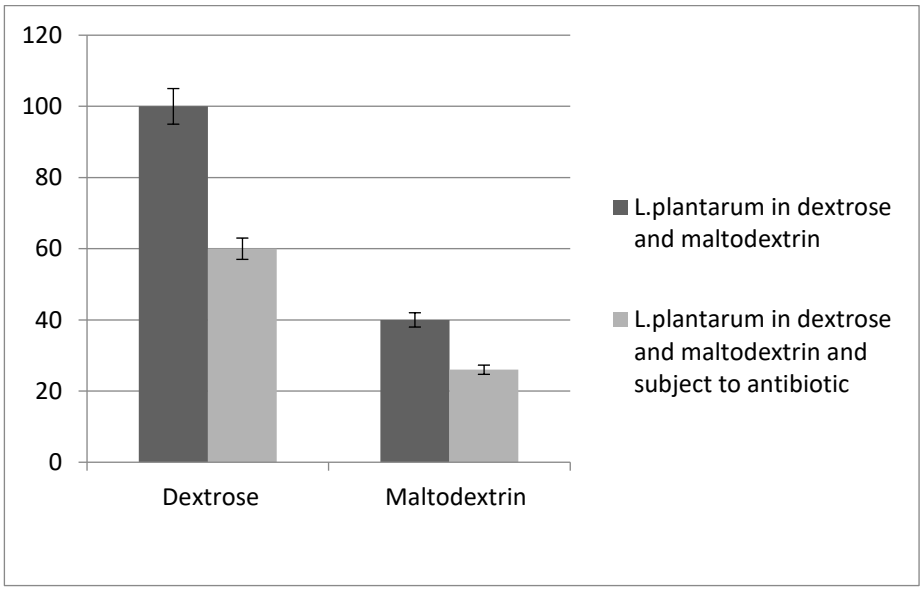
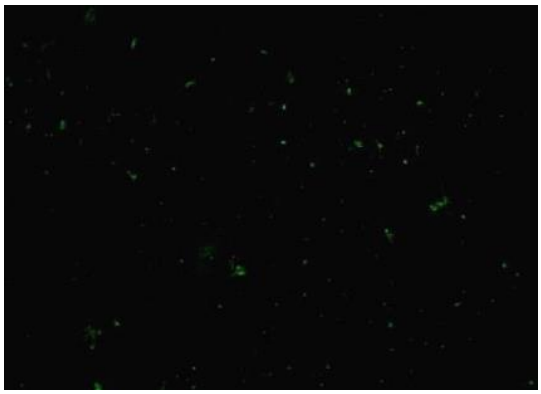


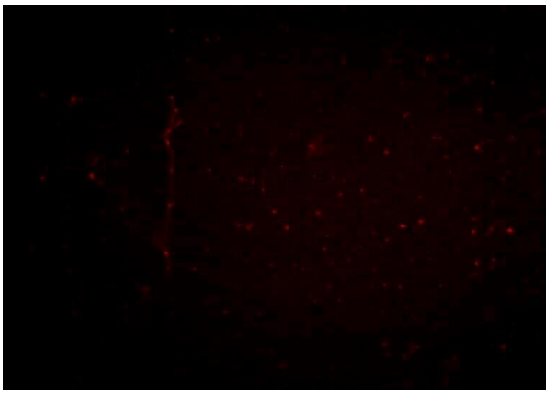
Figure 10 represents the cell viabiliy of *L. plantarum* towards the antibiotic in presence of dextrose and maltodextrin.

The cell viability of *L. plantarum* in dextrose was considered as 100% which was reduced to 40% in presence of maltodexrin. Treatment with Azithromycin reduced the viability of *L. plantarum* to 60% and 25% in dextrose and maltodexrin respectively. The cell viability of *L. plantarum* decreased hugely in presence of maltodextrin and made the cells sensitive to ampicilin. To check the membrane integrity of the cells, MTT assay was performed.

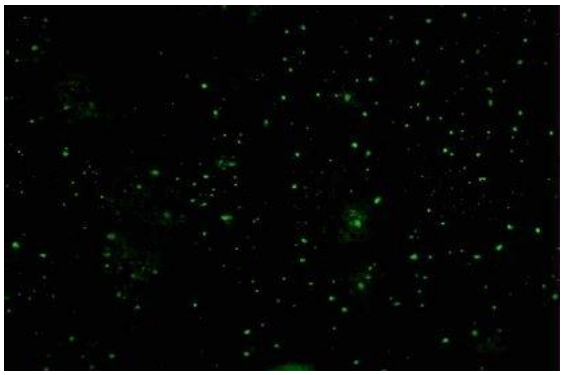
3.3.12. Fluoroscence microscopic view of *Lactobacillus rhamnosus* 5300 and *Lactobacillus plantarum* 2083 grown in dextrose and maltodextrin



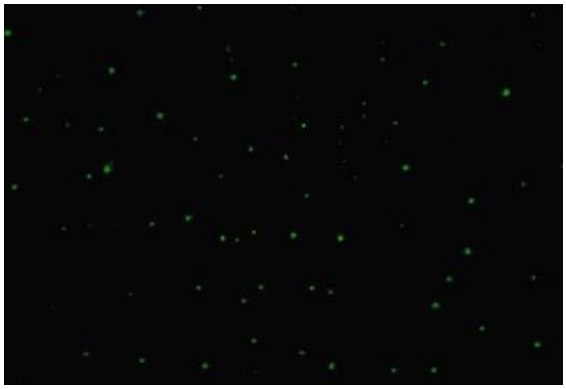
A. view of *L. plantarum* in dextrose



B. view of *L. plantarum* in maltodextrin



C. view of *L. rhamnosus* in dextrose



D. view of *L. rhamnosus* in maltodextrin

Figure 11

Figure 11 represents *L. plantarum* and *L. rhamnosus* grown in presence of dextrose and maltodextrin and stained with acridine orange and propidium iodide. Acridine orange is an intercalating dye which penetrates both live and dead cells and genetrates green fluoroscence. Propidium iodide enters inside the perforated cells and both the dyes together produce a red coloration. Hence, intact cells look green and perforated or dead cells look red. The pictures represent the average trend over 20 fields.

Figure 11A represents *L. plantarum* grown in dextrose and having intact cellular membrane structure as they appear as green.

Figure 11B represents *L. plantarum* grown in presence of maltodextrin and appear red depicting perforated cellular membrane structure.

Figure 11C represents *L. rhamnosus* in dextrose and appear green. This represents the intact cellular structure and membrane integrity of *L. rhamnosus*.

Figure 11D shows *L. rhamnosus* grown in maltodextrin and appear as green representing the intact cellular membrane.

The results showed that *L. plantarum* when grown in maltodextrin lost its membrane integrity. This result explains the previous two observations i.e, increased antibiotic sensitivity and less cell viability of *L. plantarum* in presence of maltodextrin.

4.5 **DISCUSSION**

The results clearly showed that all the oligosaccharides do not act as prebiotics for all probiotics. Fructooligosaccharides and maltodextrin acted as the best prebiotic for *L. casei*. The dose of the prebiotic is also very important as high concentration of prebiotic increases the osmolarity of the medium and thus inhibits the growth of the microorganisms. Results suggest that FOS acts as the best prebiotic for *L. casei* whereas it had no prebiotic effect on *L. rhamnosus*. Maltodextrin acts as a prebiotic for *L. casei* and *L. rhamnosus* compared to FOS. Inulin facilitates the growth of *L. plantarum*.

The presence of maltodextrin in media showed to reduce the MIC values of the antibiotic ciprofloxacin for *L. casei* and *L. rhamnosus* but had no effect on *L. plantarum*. *L. rha mnosus* and *L. casei* showed increased susceptibility towards azithromycin in the presence of maltodextrin whereas *L. plantarum* showed less sensitivity. It can be concluded that *L. plantarum* in combination with the prebiotic maltodextrin can be used in case of ciprofloxacin treatment**.**

On the other hand, the sensitivity of *L. plantarum* towards ciprofloxacin increases in the presence of inulin. Hence *L. plantarum* should not be used in presence of ciprofloxacin. *L. rhamnosus* showed same level of inhibition both in inulin and dextrose. Similarly, *L. plantarum* showed increased sensitivity towards ampicillin when grown in maltodextrin compared to dextrose while *L. rhamnosus* showed similar sensitivity in both dextrose and maltodextrin.

Based on the above results, studies were focused on the probiotic *L. casei* and the change of pH was checked for both MRS broth (with dextrose) and modified MRS broth (with maltodextrin). The pH of the growth media containing maltodextrin was found to decrease to 3.5±0.008 after 24 hours compared to the pH of 4.9$\pm$0.009 in the dextrose containing growth media. SEM studies showed decrease in size of the *L. casei* when grown in presence of maltodextrin. Thus, though growth rate of *L. casei* increases in the presence of maltodextrin and more cells are generated, the size of the cells is getting shorter. This observation was further supported by the MTT assay where less cell viability was observed in presence of maltodextrin compared to dextrose. The cells lost viability more after Azithromycin treatment compared to dextrose. The pH of the dextrose containing media was decreased to 3.5 to check

the effect of low pH on *L. casei* cellular morphology. However no remarkable changes were observed on cellular morphology as found in maltodexrin. Moreover, all the experiments were done with *L. casei* culture grown in dextrose and maltodexrin for 3 hrs. The pH of the growth media did not go down during that time. Hence change in pH is not the reason behind altered cellular morphology and increased permeability. Hence maltodextrin cannot be used as an effective prebiotic or growth simulator for *L. casei* MTCC 1423 in presence of Azithromycin.

Similarly, it was found that *L. plantarum* loses its viability in presence of maltodextrin compared to dextrose whereas *L. rhamnosus* showed almost the same level of responsiveness in both maltodextrin and dextrose**.** MTT assay and Fluorescence microscopy results showed that *L. plantarum* cell membrane gets damaged when grown in presence of maltodextrin compared to dextrose. Similar changes were not observed with *L. rhamnosus* when grown in maltodextrin.

Hence, the sensitivity of the probiotics in presence of prebiotics and antibiotics varies considerably. Hence, choice of a prebiotic and probiotic for better synbiotic effect is very important for any treatment and particularly along with antibiotics.

3.6 CONCLUSION:

- All the three prebiotics fructooligosaccharide, maltodextrin and inulin increased the growth rate of *L. casei*, *L. rhamnosus* and *L. plantarum*. However, the response of the lactobacilli was found to vary against antibiotics in presence of these prebiotics.
- *L. casei* became highly sensitive to Azithromycin in presence of maltodextrin compared to dextrose.
- *L. plantarum* showed increased sensitivity towards ciprofloxacin and ampicillin when grown in inulin and maltodextrin respectively while *L. rhamnosus* showed no such effects.
- *L. casei* and *L. plantarum* lost their cell viability considerably in presence of Azithromycin and ampicillin in presence of maltodextrin
- *L. rhamnosus* showed very little change in metabolic activity when grown in maltodexrin.
- SEM analysis revealed shortening of *L. casei* in presence of maltodexrin.
- *L. plantarum* lost its membrane integrity when grown in presence of maltodextrin as revealed from fluorescence microscopic pictures.
- Further studies on the interaction and mechanism of action of maltodextrin on *L. casei* would throw some light on the reasons behind the increased sensitivities of a particular probiotic in presence of a prebiotic.

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CHAPTER 4

Role of *Saccharomyces boulardii* on different physiological parameters

4.1 INTRODUCTION

The beneficial properties of *Saccharomyces boulardii* are known for years and have been well documented in literatures (McFarland et al, 1994). Administration of it in combination with antibiotics has shown to decrease the duration and the frequency of diarrhea significantly (Zanello et al, 2009). It can serve as a potent mean to control diseased conditions like acute diarrhea, recurrent infection caused by *Clostridium difficile*, irritable bowel syndrome, travelers' diarrhea and diarrhea associated with antibiotic therapy or with HIV/AIDS. *Saccharomyces* spp. also has an antagonistic effect on *Candida albicans* infection (Krasowska et al, 2009).

It is known that *S. boulardii* secretes a 54 kDa protease, which degrades toxins A and B of *Clostridium difficile* in vivo. The protease also inhibits the binding of the toxins to the receptors present on the brush border and thus reduces the enterotoxic and cytotoxic effects of the infection **(**Castagliuolo et al, 1996). Another 120 kDa protease, secreted by *S. boulardii* modulates the activity of enterocytes present in the lining of the large and small intestine. It leads to the reduction in enterocytic cAMP production and chloride secretion and thus reduces the excess secretion of water and electrolytes as in the case of *Vibrio cholerae* infection **(**Czerucka et al, 1989). The lectin receptors present on the membrane of *Escherichia coli* and *Salmonella typhimurium* interact with the mannose residues found on the surface of *S. boulardii* and prevent the attachment of these bacteria to the intestinal brush border. These cellular aggregates are then eliminated from the body through bowel movement. The production of anti-inflammatory cytokine IL-10 and reduction in production of pro-inflammatory cytokines like TNF-α and IL-6 have been found in *S. boulardii* **(**Sougioultzis et al, 2006). Increased production and secretion of Immunoglobulin A (IgA) has been found in the small intestine of the rat fed with *S. boulardii* which provides protection against invading microbes in the gastrointestinal and respiratory tracts (Buts et al, 1990). It also activates the innate as well as the humoral branch of immune system in gnotobiotic mice (Rodrigues et al, 1996).

Polyamines

Natural polyamines like spermidine, spermine and putrescine, are small organic cationic molecules having distinct cellular functions and essential for the control of multiple signaling pathways in eukaryotic cells. Polyamines regulate intestinal epithelial integrity by maintaining Normal intestinal epithelial growth and regulating intestinal epithelial cell apoptosis (Kibe et al, 2014). The concentrations of polyamines decrease in somatic tissues including the brain with increasing age. It has been found that foods rich in polyamines enhance longevity and provides protection from age-induced memory impairment in several living model system. Chronic low-grade inflammation, an important factor that contributes to senescence and age-related diseases, is known to be decreased by polyamines. They act by inhibiting the synthesis of inflammatory cytokines in macrophages. Thus reductions in intestinal luminal polyamine levels have been associated with intestinal barrier dysfunction. Other important functions mediated by polyamines include interaction between receptor and ligand, functioning of ion channels, stability of membranes etc.. Polyamines play a major role in liver regeneration by stabilizing DNA, modulation of chromatin structure etc (Okumura et al, 2016).

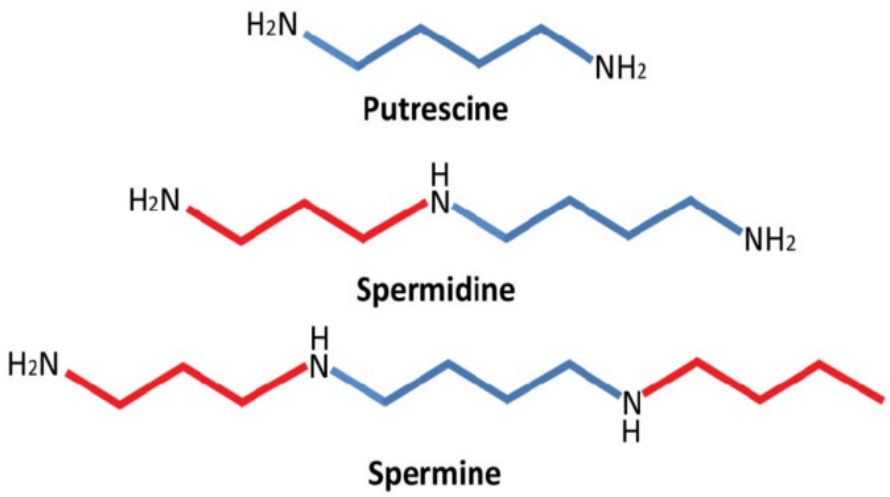


Figure: Structures of polyamines

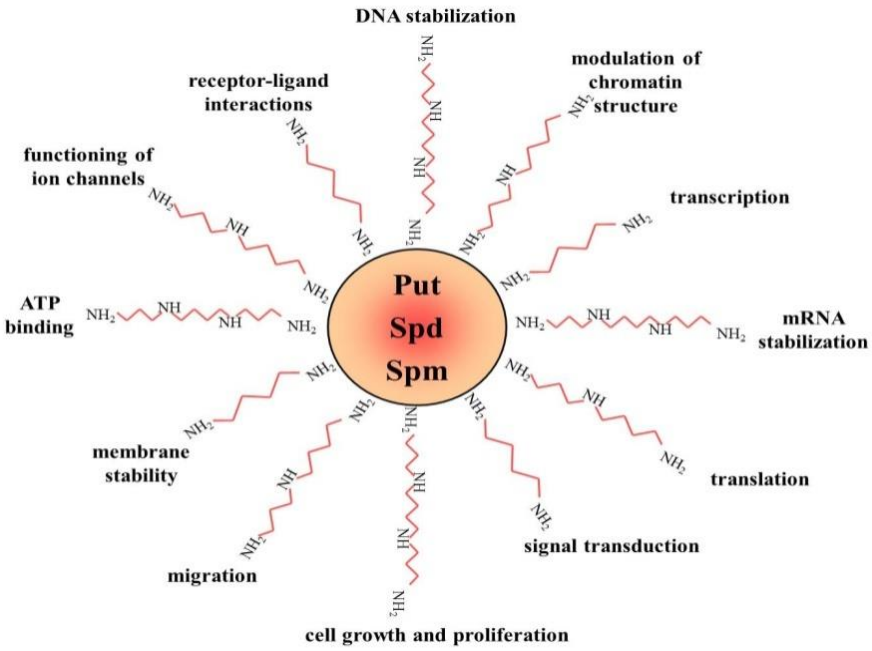


Figure: Functions of polyamines in a cell

The intracellular polyamine pool is contributed by several factors e.g, de novo biosynthesis of polyamines, diet rich in polyamines and gut microflora. Intestinal microflora contributes a considerable amount of polyamines in the gut. Arginine intake increases the concentrations of polyamines in the colon and blood. Combinatorial administration of arginine and bifidobacteria LKM512 to mice has shown to suppress inflammation, improved longevity, and provides protection from age-induced memory impairment (Kibe et al, 2014). In another study, the authors showed that the same probiotic strain increased intestinal luminal PA concentrations (Matsumoto et al, 2011).

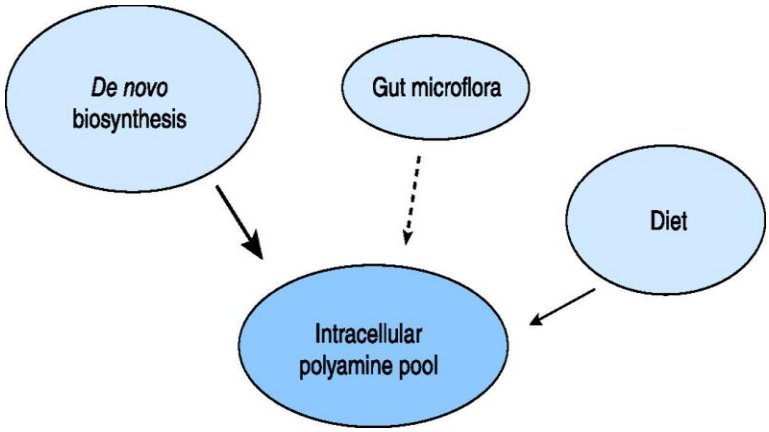


Figure: Source of polyamines in cells

Liver regeneration is also accompanied by a significant increase in fasting putrescine and spermidine concentrations (Marchesini et al, 1992). Another study showed that spermidine and/or spermine, but apparently not putrescine, are required for liver regeneration after partial hepatectomy (Alhonen et al, 2002). A recent study shows that perioperative administration of polyamine orally attenuates liver ischemia/reperfusion injury (IRI) and promotes liver regeneration (Okumura et al, 2016). Hence, administration of polyamines at the desired dose might be a new therapeutic option to improve the outcomes of partial liver transplantation.

4.2 OBJECTIVE

- Investigation of the hepatoprotective function of *Saccharomyces boulardii* on CCl_4 induced acute liver damage in swiss albino mice.
    - Estimation of SGPT, ALP, total protein
    - Estimation of lipid peroxidation in liver tissues
    - Histological studies
- Investigation of the role of *Saccharomyces boulardii* on kidney function in mice after CCl_4 challenge.
    - Estimation of blood urea nitrogen, creatinine,
- Finding out the concentrations of polyamines in liver before and CCl_4 challenge.
    - Estimation of spermine, spermidine, putriscine
    - Estimation of total polyamine

4.3 MATERIALS AND METHODS:

4.3.1. PROBIOTIC STRAIN:

Saccharomyces boulardii, a member of the family saccharomycetaceae, was a gift in lyophilized form from Prof. Mercin Lukazwick of University of Wrocklaw, Poland.

Another source of Saccharomyces boulardii was "ECONORM", a product manufactured by Dr. Reddy's laboratory, Hyderabad, India.

4.3.2. ANIMALS

Young male Swiss albino mice (body weight 20±2 gm) were used for the animal studies. The mice were acclimatized in the lab for two weeks before any experiment and were fed food and water ad libitum. The mice were kept fasted for 16 to 18hrs before performing any experiment. All the studies were conducted in accordance with the guideline of standard experimental animals study ethical protocols. All the studies were done in Bose institute, Kolkata.

4.3.3. CHEMICALS

CCl_4, TBA, TCA and all other chemicals were purchased from Sisco research laboratory INDIA. The kits for the measurement of ALT, ALP, BUN, creatinine were procured from Span diagnostics India. The protein estimation kit was procured from BANGALORE Genei private Ltd. All the chemicals were reagent grade of the highest laboratory purity.

4.3.4. BIOASSAY

There were 4 groups of mice with 6 mice in each group. They were given food and water ad libitum. Mice were intoxicated by CCl_4 by oral gabage.

Group 1: Normal control

Group 2: Toxin control mice got hepatotoxin CCl_4 only, orally at a dose of 1ml/kg body weight along with liquid paraffin on 6^{th} day.

Group3: Mice got *S. boulardii* in two ways in separate experiments:

i) at a dose of 1gm/kg body weight for 5 consecutive days

ii) 10^7 cells/mouse with food once daily for 5 consecutive days. The mice were given CCl_4 on the 6^{th} day at the same dose as group 2.

Group 4: The mice of this group received *S. boulardii* only as the same dose as group 3.

4.3.5. Measurement of hepatotoxicity

The mice were sacrificed after 48 hrs of CCl_4 administration and samples of blood were collected by puncturing the hearts. The blood samples were kept overnight and centrifuged at 3000 rpm for 10mins to separate the serum. The levels of serum ALT, ALP, BUN and Creatinine were determined spectrophotometrically according to the standard protocols.

4.3.5.1. Estimation of serum glutamate pyruvate transminase (SGPT)

Principle

α-ketoglutarate + L-alanine ↔ L-glutamate + Pyruvate

Pyruvate+ 2, 4 dinitrophenyl hydrazine → hydrazone

The hydrazone produces a brown colour in alkaline medium and optical density is measured at 505 nm.

Procedure

0.25 ml of buffered alanine-α-ketoglutarate substrate was incubated at 37ºC for 5 minutes. Then 0.05 ml serum was mixed into it and incubated for 30 minutes at the same temparature. DNPH colour reagent (0.25 ml) was added, mixed well and allowed to stand at room temperature (RT)

for 20 minutes. Finally, 2.5 ml NaOH solution was mixed, kept at RT for 10 minutes and the O.D was taken at 505 nm (Reitman and Frankel, 1957).

Preparation of standard curve: A standard curve was constructed by plotting enzyme activity (different concentrations of standard pyruvate were used) vs absorbance at 505 nm. The enzyme activity of the unknown sample was determined from the curve.

4.3.5.2. Estimation of serum alkaline phosphatase (ALP)

Principle

Phenyl phosphate → Phenol+Pi

Phenol+4-amino antipyrine----------→ orange red complex

The enzyme alkaline phosphatase present in the serum cleaves the phosphoryl group from phenyl phosphate to produce inorganic phosphate and phenol at pH 10. Phenol so formed reacts with 4-aminoantipyrine in alkaline medium in presence of the oxidizing agent potassium ferricyanide and forms an orange-red coloured complex, which is measured colourimetrically at 510 nm. The colour intensity is proportional to enzyme activity.

Procedure

Working buffered substrate (0.5 ml) was mixed with 1.55 ml distilled water and incubated for 3 minutes at 37ºC. Then 0.05 ml serum and 0.05 ml phenol standard was mixed into test and standard measurement respectively. After incubation of 15 minutes at 37ºC, 1.0 ml chromogen reagent was added to it. For the measurement of control, 0.05 ml serum was added at the end. The O.D was taken at 510 nm (Kind and King, 1954).

Serum ALP activity in KA units is as follows: $\frac{\text{O.D Test} - \text{O.D Control}}{\text{O.D Standard} - \text{O.D Blank}} \times 10$

4.3.5.3. Estimation of protein

Protein concentration was estimated according to the method of Bradford (Bradford, 1976). BSA was taken as standard protein.

A standard curve was drawn with known concentrations of BSA and the unknown concentration was determined from the standard curve.

4.3.5.4. Histological Studies

Tissue processing-Immediately after removal from body, the liver tissues were fixed in 10 % formalin for 2-4 hrs. The excess fixative was removed by washing with water.

Dehydration-The tissues were next passed through a series of graded alcohols. Dehydration time was 1 hr in 70% alcohol, 1 hr in 80% alcohol, 2 hrs in 95% alcohol and 3 hrs in absolute alcohol.

Clearing-The process of clearing involves the removal of alcohol and other dehydrating agents. After removing the tissue from the last bath of absolute alcohol, was placed in xylene for ½ to one hour and then transferred to wax.

Impregnation-After clearing, the tissues were infiltered with a supporting medium to make the tissue firm which facilitates easy sectioning and keeps the various components of the tissue in proper relation. The tissues were first placed in the first paraffin wax bath for 2 hrs and then in the second bath for 2 hrs.

Embedding or blocking- Embedding is the process of placing the impregnated tissue in a precisely arranged position. The tissues were lifted from the final wax bath and pressed to the bottom of the embedding medium with the cutting surface facing downwards.

Technique of cutting paraffin embedded section-The block was fixed on block holder and clamped on the microtome apparatus. The microtome was operated till complete sections are cut

(5 μm) and the ribbon was formed. The sections were floated on a water bath having temperature 43-47^{o} C to remove the wrinkles.

Mounting of sections- The egg white (albumin) and glycerine were mixed together to make an adhesive and a thin smear was formed over the slide. The albuminized slides were immersed in the water and the sections were brought on to the centre of the slide. The slides were kept vertically to drain out the water. The slides were kept at 62^{o}C for few seconds to melt the wax. After melting the wax, the slides were put in xylene for 10 minutes to wash off the wax and then air dried for 1 minute.

Staining- The haematoxylin and eosin staining technique is commonly used to stain tissue sections. Haematoxylin being a basic stain is taken by the nucleus and the surrounding is counter stained by eosin. The slides were first put in haematoxylin stain for 3-5 minutes and kept in running tap water for a few minutes. Then the slides were immersed in 1 % and 2 % HCl respectively and again kept in running tap water for 30 minutes. Then the slides were dipped in eosin stain for a few seconds. The excess stain was washed off by 90 % alcohol, dipped in xylene and placed for mounting.

Mounting- One drop of mounting media, D.P.X (distyrene, tricresyl phosphate and xylene) was placed on the coverslip. The coverslip with D.P.X was gently lowered on the section. D.P.X spreads evenly and excess of it was wiped off. The slides were ready for examination under microscope.

4.3.5.5. Measurement of Malondialdehyde (MDA) in liver tissue

Principle

Malonaldehyde, the dialdehyde of malonic acid, produced as a result of lipid peroxidation and thus a biomarker of oxidative damage. Malonaldehyde reacts with thioberbituric acid (TBA) and produces a complex, which is measured colorimetrically at 535nm. One molecule of MDA reacts with two molecules of TBA with the production of a pink pigment when heated at 90-100^{o}C for 10-15 min at a pH of 2-3. It has been found that 99.7 % of the absorbance at 535 nm of TBA assay results from MDA and only 0.3 % absorbance are due to other aldehydes (Esterbauer and Cheeseman, 1990).

Procedure

0.2gm of liver tissue was homogenized in 20 mM phosphate buffer (pH 7.4) and centrifuged at 3,000 g for 10 minutes at 4˚C. The supernatant containing malonaldehyde was mixed with twice volume of TCA-TBA mixture (15 % W/V TCA, 0.375 % W/V TBA) and heated at 100˚C for 15-30 mins. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 minutes. The absorbance of the sample was determined at 532 nm against a blank that contains all the reagents except the liver sample. The malonaldehyde conc. of the sample was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

4.3.6. Measurement of kidney toxicity

Blood urea nitrogen (BUN) and creatinine were measured as markers of kidney because the ammonia produced in liver is excreted through kidney. BUN is also a marker of liver.

4.3.6.1. Estimation of Blood urea nitrogen

The level of BUN in serum was determined by diacetylmonoxime (DAM) method (Van Slyke and Cullen 1914).

Principle

Urea + hot acidic DAM + thiosemicarbazide → rose-purple coloured complex

Procedure

10µl of serum was mixed with 2.5ml of urea reagent containing thiosemicarbazide and 250ml of DAM and mixed properly. The tubes containing reaction mixtures of test sample, standard and control were kept in a boiling water bath for exactly 10 minutes followed by immediate cooling. The optical density of the coloured complex formed in each tube was measured spectrophotometrically at 525 nm.

Serum urea in mg / dl (A) = (O.D of Test/ O.D of Standard) × 30

Blood Urea Nitrogen (BUN) in mg / dl = (A) X 0.467

4.3.6.2. Determination of serum Creatinine

The level of creatinine in serum was measured by following the alkaline picrate method of Bonses and Taussky (1945).

Principle

Creatinine + alkaline picrate ⟶ red coloured complex

Procedure

Serum, distilled water and picric acid were mixed in 1:1:6 ratio and was placed in a boiling water bath exactly for one minute and then centrifuged in 3000 rpm for 10 minutes. The O.D of the supernatants collected from the blank, test and standard were measured at 520nm (Toora & Rajagopal et al, 2002).

Serum Creatinine in mg/dl = (O.D Test-O.D Blank)/(O.D. Standard – O.D. Blank) x 3

4.3.7. Estimation of blood Cholesterol

The concentration of cholesterol in blood was estimated by ferric chloride-sulfuric acid method (Wybenga et al, 1970).

Principle

Cholesterol + hot solution of ferric per chlorate, ethyl acetate and sulphuric acid ⟶

Lavender colored complex

Procedure

The serum and the reagents were mixed together and all the tubes were kept in the boiling water bath exactly for 90 seconds. After cooling, O.D of all the samples was taken at 560 nm.

Calculation: Total Cholesterol (mg/dl): [(O.D of Test)/ (O.D of Standard)] X200

4.3.8. Estimation of Spermidine

The concentration of spermidine in liver extract was estimated by Bachrach and Oser.

Principle:

Spermidine + ortho aminobenzaldehyde ⟶ yellow colored complex

Procedure:

Liver samples were homogenised in PBS buffer and 0.01-0.3µl of liver homogenate was added to 0.5ml of 50 pmoles of sodium phosphate buffer, pH 6.6 and 0.1ml of sodium chloride solution (0.15M) to make a final volume of 0.9 ml. This suspension was incubated at 37ºC for 30 mins. 1ml of 0.1x ortho aminobenzaldehyde sodium was then added and the reaction mixture was incubated at 37ºC for another 30 mins. The intensity of the yellow colour was then determined spectrophotometrically at 435nm against proper control (sodium chloride). The molar extinction coefficient of the Pyrroline O aminobenzaldehyde complex is 1.8610 litre/mole .

4.3.9. Estimation of Spermine

The concentration of spermine in liver extract was estimated following the method of Miyachi et al. (Miyachi et al 2007)

Principle:

Spermine + o-hydroxyhydroquinonephthalein (QP) $\longrightarrow$ Mn (II)-QP-Spm

Procedure:

Samples of liver homogenates were mixed with 2.0 ml of the tris buffer solution, 2.0 ml of 1.0% Tween 40 solution, 0.5 ml of a 1.0×10^{-3}M Mn (II) solution and 0.5 ml of a 1.0×10^{-3}M o-hydroxyhydroquinonephthalein (QP) solution. The reagents were mixed well and kept at room temperature for 15 min. The absorbance of the ternary complex formed between Mn (II)-QP-Spm (1:2:2) was measured at 555 nm against a reagent blank solution.

4.3.10. Estimation of Putrescine

The concentration of putrescine in liver extract was estimated following the method of Ngo et al.

Principle:

Putrescine + 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) + 1-pentanol $\longrightarrow$ Coloured complex

Procedure:

0.5ml of samples of liver homogenate were mixed with 1ml of 4N NaOH and mixed vigorously. 2ml of 1-pentanol was then added and the samples were mixed properly. The emulsions were centrifuged for 5mins at 2000rpm and 1ml of the organic phase was mixed with 1ml of 0.1M sodium borate, pH 8.0. 1ml of 10mM TNBS (dissolved in 1 pentanol) was added to each tube and mixed properly. 2ml of DMSO was added and mixed properly. The phases were separated by centrifugation at 2000rpm for 5 mins. The absorbance of the organic phase was measured against a reagent blank at 420 nm (Ngo et al, 1987).

4.3.11. Estimation of total polyamine

The concentration of total polyamine was estimated by High performance liquid chromatography. Liver homogenates of all the samples, normal, CCl_4 treated, *S. boulardii* pretreated, were prepared in 5% perchloric acid and diluted to obtain the necessary final concentrations. 50µl of each sample was taken in vials containing saturated sodium carbonate solution and 100µl volume of dansyl chloride solution in acetone (10 mg/ml) was added to each of them. The vials were incubated in a water bath at 60°C for 1 hr in the dark. Next, excess dansyl chloride was removed by the addition of 50µl of proline (100 mg/ml) to the reaction mixture and incubated for 30 mins. Next step was evaporation of acetone from each vial and addition of 400 ml toluene to each vial. After proper mixing, the vials were centrifuged at 500 g for 2 mins and 200ml of the toluene layer were transferred to Eppendorf tubes. After complete evaporation of toluene, the residue was dissolved in 1 ml of methanol or acetonitrile. The samples were chromatographed using a C_{18} reversed-phase cartridge column and a linear gradient of acetonitrile-heptanesulfonate (10 mM, pH 3.4) at a flow-rate of 2.5 ml/min was maintained **(**Minocha et al, 1990).

Statistical analysis

The results have been represented as the mean ± SD. *P* values less than 0.05 (two-tailed) were considered statistically significant.

4.4 RESULTS

4.4.1 Effect of CCl_4 and *S. boulardii* on the ALT level in mice serum

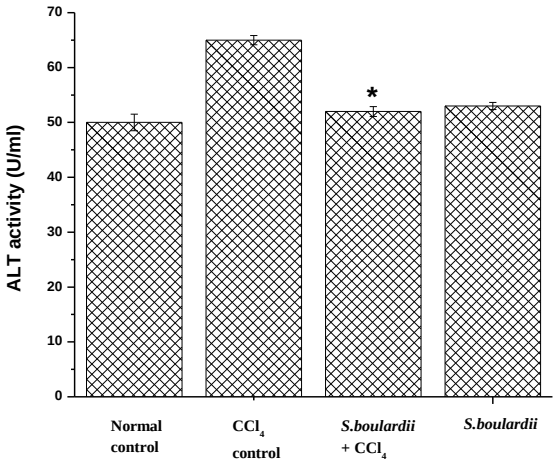


FIGURE 1

Normal Cont: normal control mice
CCl_4 treated: CCl_4 treated mice
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates the significant decrease in ALT activity in mice treated with *S. boulardii* prior to CCl_4 administration compared to ALT activity in CCl_4 treated mice only. ($P^{*}<0.001$)

The effects of pretreatment of *S. boulardii* on ALT activity in all four groups of mice have been shown in figure 1. The ALT activity was found to increase (65.5$\pm$1.8 U/ml of serum) in the serum of mice treated with CCl_4 compared to the activity of ALT (50$\pm$1.6 U/ml) in normal serum after 48 hrs of CCl_4 administration. The serum ALT level was much less (52$\pm$0.9 U/ml) in the mice received *S. boulardii* for 5 days before CCl_4 administration at a dose of 1gm/kg body weight. Hence, it can be said that presence of *S. boulardii* significantly prevented the rise of serum ALT activity. The application of *S. boulardii* alone had no effect on serum ALT activity.

4.4.2. Effect of CCl_4 and *S. boulardii* on the ALP level in mice serum

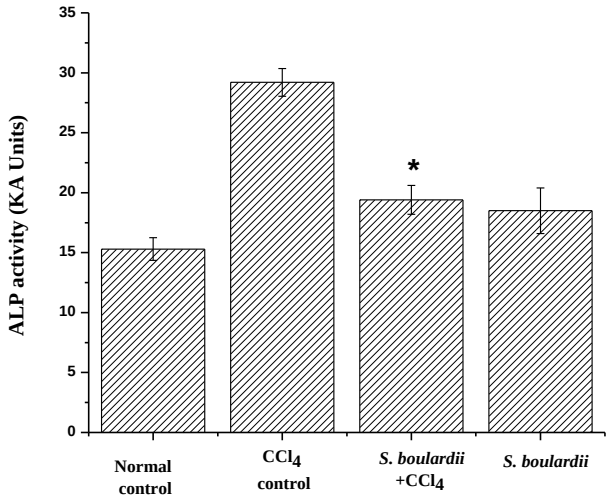


FIGURE 2

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates the significant decrease in ALP activity compared to toxin control. ($P^{*}<0.001$)

The effects of pretreatment of *S. boulardii* on the serum ALP level in all experimental mice have been shown in figure 2. The serum ALP activity (29.2$\pm$1.16 KA U/ml) was elevated almost 2 folds than the normal serum ALP activity (15.3$\pm$0.94 KA U/ml) after 48 hrs of CCl_4 administration. The ALP activity was much less (19.4$\pm$1.2 KA U/ml) in the serum of mice received *S. boulardii* for 5 days before CCl_4 administration. Hence, treatment of mice with *S. boulardii* significantly prevented the increase in ALP activity.

4.4.3 Effect of CCl_4 and *S. boulardii* on the total protein concentration in mice serum

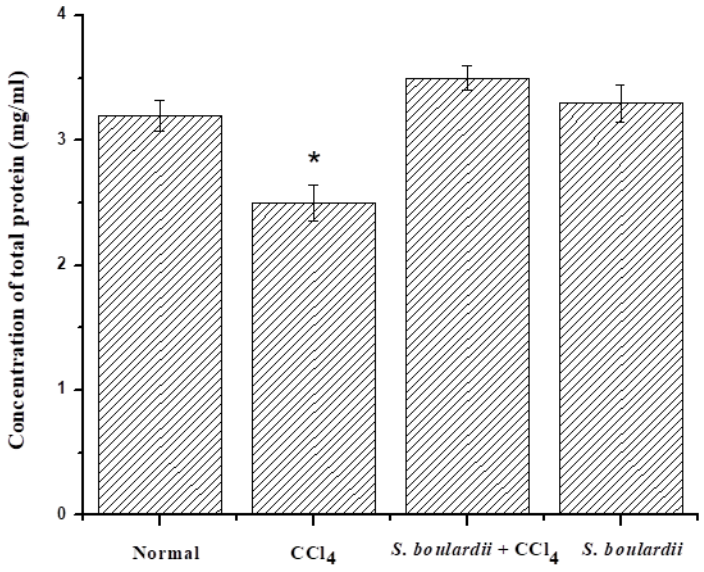


FIGURE 3

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates the significant decrease in total protein conc in mice treated with CCl_4. ((P*<0.001)

Figure 3 represents the effect of CCl_4 intoxication and effect of *S. boulardii* on the amount of total protein in mice serum. The amount of total protein in serum decreased considerably to 2.5 ± 0.14mg/ml in the group of mice treated with CCl_4 compared to the normal value of 3.2 ± 0.126 mg/ml. Interestingly, the amount of total protein was almost normal in the serum of the mice (3.5 ± 0.1mg/ml) treated with *S. boulardii* prior to CCl_4 administration.

4.4.4. Effect of CCl_4 and *S. boulardii* on the histology of liver

Histopathological samples were taken from normal, CCl_4 treated and both toxin and *S. boulardii* pre-treated mice livers. Figure 4 represents the histology of liver samples taken from all the four experimental groups of mice. Each picture is a representative of a particular group.

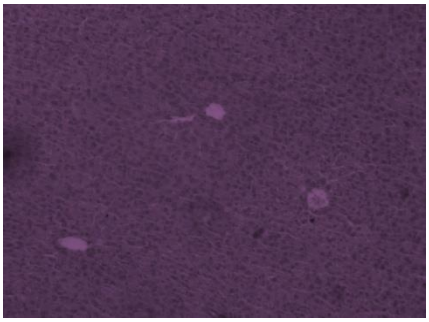
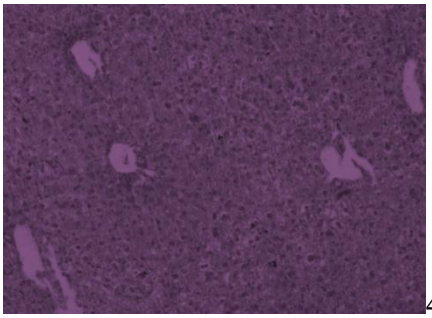
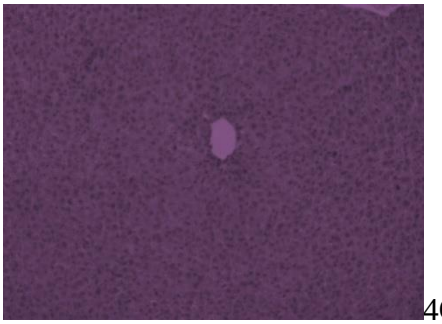
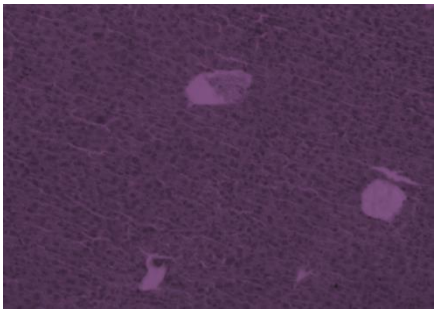

4A

4B

4C

4D

FIGURE 4: Hematoxylin and Eosin stained section (x40) of (A) Normal mouse liver (B) CCl_4 control mouse liver, a single dose (1ml/kg body weight) of CCl_4 was administered orally and the mouse were sacrificed 48 hrs later (C) *S. boulardii* pretreated mouse liver where the yeast was given at a dose of 1gm/kg body weight for 5 days before CCl_4 administration (D) Mouse liver treated with *S. boulardii* alone.

Figure 4(A) shows the liver section of normal mouse. The normal histology of mouse liver was very prominent showing the central vein, bile duct and normal hepatocytes radiating from the central vein.

Figure 4(B) shows the liver sections of CCl_4 intoxicated mice. CCl_4 caused cirrhosis in liver characterized by fibrosis, pseudolobulation, ballooning degeneration, nodule formation etc. CCl_4

also induced centrilobular necrosis around the central vein, bile duct proliferation, disruption of hepatocytes etc.

The section of mouse liver pre-treated with *S. boulardii* for 5 days before CCl_4 administration has been shown in figure 4(C). *S. boulardii* pre-treatment had very effective role in preventing the toxic effects of CCl_4 as revealed by almost normal hepatocytes and less centrilobular necrosis, bile duct proliferations etc.

Figure 4(D) shows the effect of *S. boulardii* treatment alone on liver histology. Administration of *S. boulardii* had no adverse effect on the mouse liver as revealed by intact heptocytes.

4.4.5 Effect of CCl_4 and *S. boulardii* on the extent of lipid peroxidation in mice liver

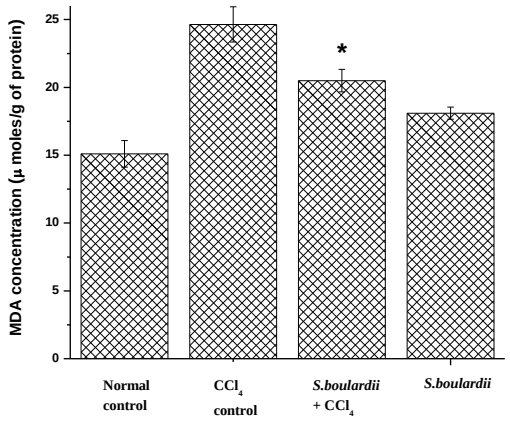


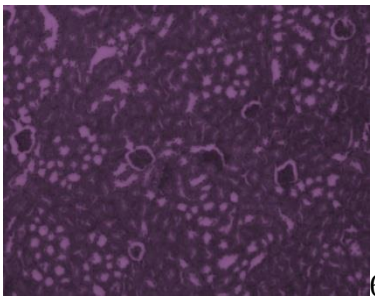
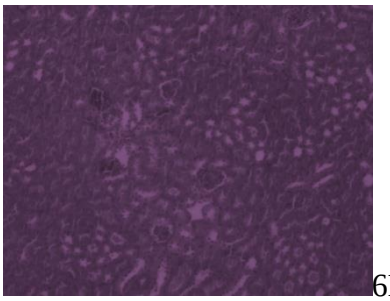
FIGURE 5

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates the significant decrease in MDA level in mice treated with *S. boulardii* prior to CCl_4 administration compared to the toxin control group. ($P^*<0.001$)

The extents of lipid peroxidation as represented by MDA in the livers of all the experimental mice have been shown in figure 5. The MDA level in CCl_4 treated mice liver (24.63 ± 1.3 moles/g of protein) increased almost 2 folds than the normal mice liver (14.1 ± 0.98 moles/g of protein). *S. boulardii* treatment for 5 days prior to CCl_4 administration prevented the damage caused by CCl_4 and hence the extent of MDA formation which is evident from low MDA level (20.5 ± 0.83 moles/g of protein).

4.4.6. Effect of CCl_4 and *S. boulardii* on the histology of kidney


6A

6B

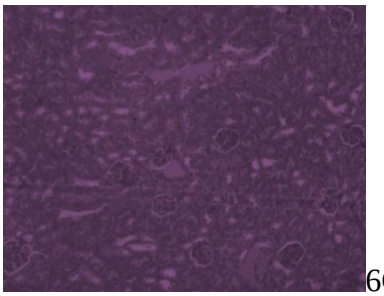
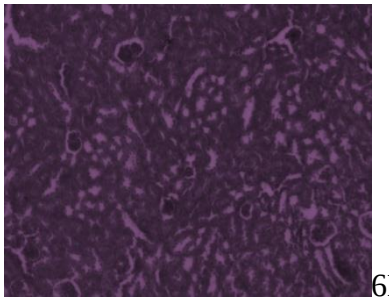

6C

6D

FIGURE 6: Hematoxylin and Eosin stained section (x40) of (A) Normal mouse kidney (B) CCl_4 control mouse kidney, (C) kidney section of mouse received *S. boulardii* (D) kidney section of mouse treated with *S. boulardii* alone.

Figure 6A shows the histology of the normal mouse kidney, Figure 6B shows the kidney section taken from mouse treated with a single dose (1ml/kg body weight) of CCl_4 orally and sacrificed 48 hrs later. Figure 6C shows the kidney section from mouse treated with *S. boulardii* at a dose of 1gm/kg body weight for 5 days before CCl_4 administration. Figure 6D shows the kidney section of mouse treated with *S. boulardii* alone for 5 days. Kidney damage was not very prominent when treated with CCl_4 and the reason behind it might be insufficient dose of the toxin and apparently *S. boulardii* had very little effect on the histology of the kidney when treated before CCl_4.

4.4.7. Effect of CCl_4 and *S. boulardii* on the blood urea nitrogen level in mice serum

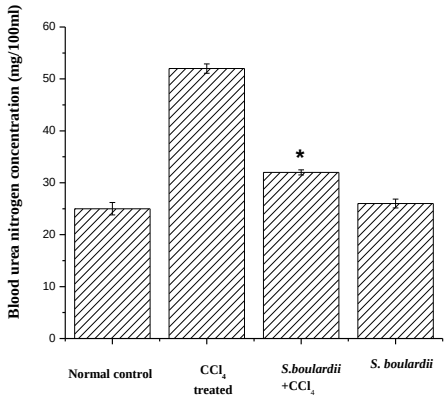


FIGURE 7

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates the significant decrease in BUN level in mice treated with *S. boulardii* prior to CCl_4 administration compared toxin control. ($P^*<0.001$)

The level of BUN is ~25$\pm$1.2 mg/100ml in the serum of normal mice and was found to increase to 52$\pm$0.9mg/100ml in the serum of CCl_4 treated mice. The level was found to decrease in mice treated with *S. boulardii* for 5 days prior to CCl_4 administration (32$\pm$0.5 mg/100ml). The BUN level was almost normal in the serum of mice received *S. boulardii* alone.

4.4.8. Effect of CCl_4 and *S. boulardii* on serum creatinine

There was no change in the serum creatinine level among the mice of four experimental groups.

4.4.9. Effect of CCl_4 and *S. boulardii* on the concentrations of total polyamines in mice liver

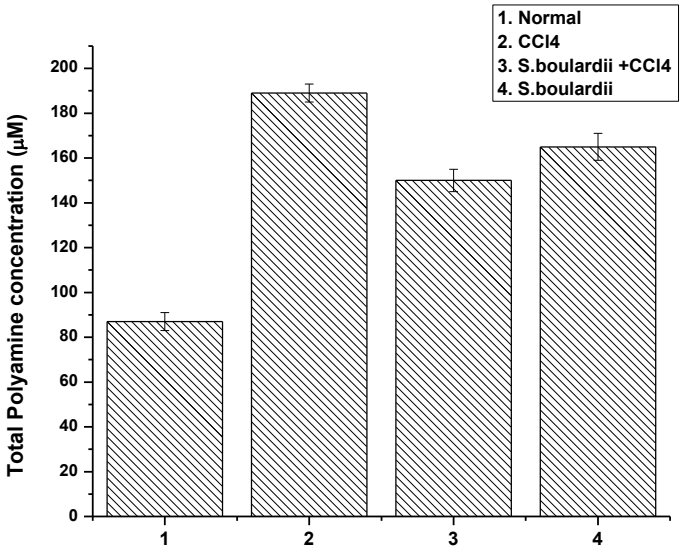


FIGURE 8

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD.

The figure shows the concentrations of total polyamines present in the livers of normal mice, CCl_4 treated mice, mice received both *S. boulardii* and CCl_4 and mice received only *S. boulardii*. The amount of total polyamine was 20µg/ml in normal mice liver and increased to 63µg/ml in CCl_4 treated mice liver due to de novo synthesis of polyamines in damaged liver. The concentration of total polyamine was much higher in the *S. boulardii* treated mice liver which might be due to the transportation of the polyamines from intestine secreted by *S. boulardii* to the liver. In case of mice, treated with *S. boulardii* prior to CCl_4 damage, the polyamine content decreased than CCl_4 intoxicated mice liver and it may account for the feedback inhibition of synthesis of putriscine.

4.4.10 Effect of CCl_4 and *S. boulardii* on the level of putriscine

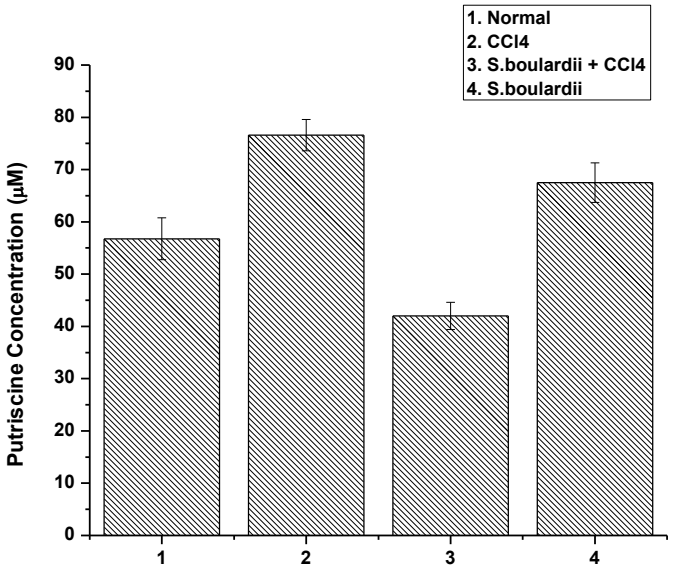


FIGURE 9

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD.

The above figure shows the effects of *S. boulardii* pretreatment to CCl_4 intoxication on the putriscine level in liver tissue. Putriscine concentration in the liver of normal group of mice was found 57 μM, which increased to 75μM in CCl_4 treated mice liver. The concentration of Putriscine in mice liver treated with *S. boulardii* prior to ccl4 intoxication was much less-40 μM. This value is lower than the value of putriscine found in normal mice liver. The concentration of putriscine was found to increase to 70 μM in the livers of mice treated with *S. boulardii.*

4.4.11. Effect of *S. boulardii* pretreatment on spermidine activity in CCl_4 induced liver damage in mice

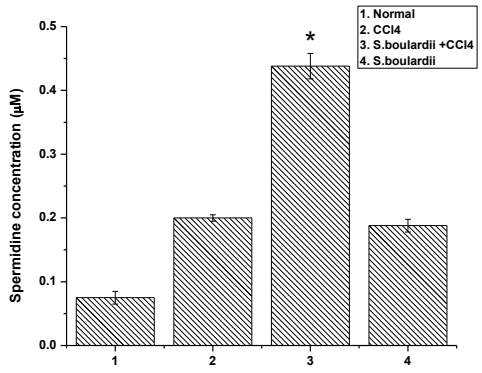


FIGURE 10

Normal Cont: normal control mice;
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates the significant increase in spermidine activity in mice treated with *S. boulardii* prior to CCl_4 administration compared to the spermidine activity in normal and CCl_4 treated mice only. ($P^*<0.001$)

The above figure shows the effects of *S. boulardii* pretreatment to CCl_4 intoxication on the spermidine level in liver tissue. Spermidine concentration in the liver of normal group of mice is 0.075 μM which is found to be increased to 0.2 μM in CCl_4 treated mice liver. The concentration of spm was found to increase (almost same as in the livers of CCl_4 treated mice) in the livers of mice treated with *S. boulardii*. More interestingly, spermidine concentration was found to increase more than 5 fold (0.43 μM) compared to normal in *S. boulardii* pre-treated mice livers. This increase in spm might be due to the de novo biosynthesis of Spm in damaged liver in addition to the polyamines coming from *S. boulardii* (Zhang et al 2000).

4.4.12. Effect of *S. boulardii* pretreatment on spermine activity in CCl_4 induced liver damage in mice

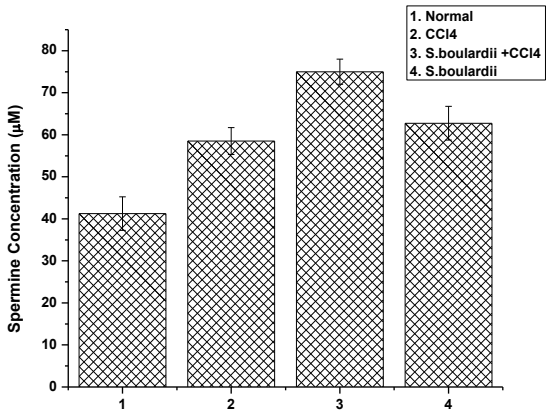


FIGURE 11

Normal Cont: normal control mice
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD.

Spermine concentration in the liver of normal group of mice was around 40Micromole which was found to increase to 60 µM in CCl_4 treated livers of mice.The concentration of spermine was found even greater in S.boulardii pre treated mice liver and was-75 µM. *S.boulardii* treatment alone was also found to increase the concentration of spermine in mice liver. This increased value is might be due to de novo SPM synthesis in damaged liver along with *S. boulardii* induced polyamine synthesis. It is already known that SPM concentrations significantly increase in tissues following injury, inflammation, and antigen stimulation, attributed to both its release from dying and injured cells and its stimulated biosynthesis (Zhang et al, 2000). The concentration of spm was found to increase (almost same as in the livers of CCl_4 treated mice) in the livers of mice treated with *S. boulardii.*

4.4.13. Correlation between ALP and Putriscine levels in damaged liver

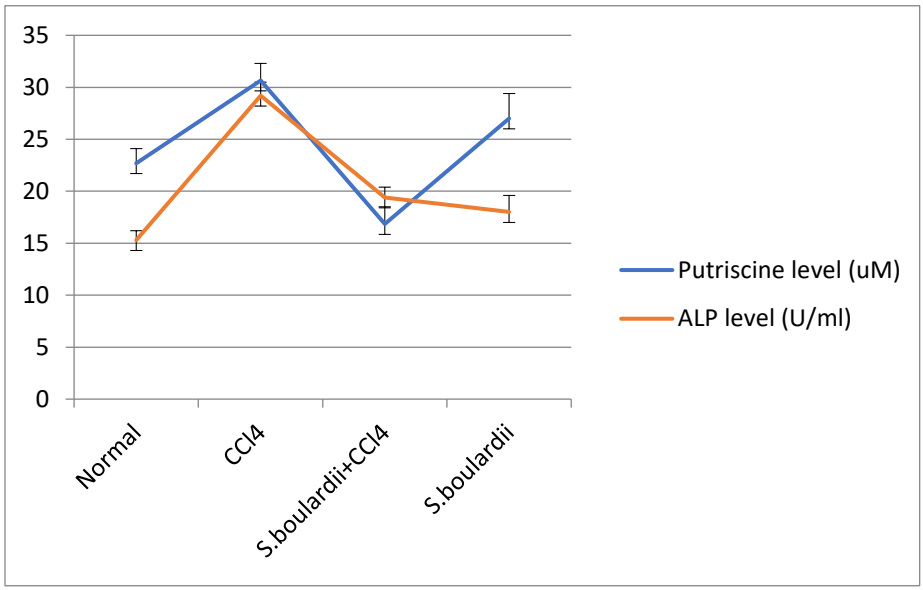


FIGURE 12

The levels of ALP i.e the marker for liver damage and the level of Putriscine, precursor of polyamines required for liver regeneration are in positive correlation. Both the levels of Putriscine and ALP increase in CCl_4 treated mice liver and decrease in the mice liver pre-treated with *S. boulardii*. Decrease in Putriscine concentration in *S. boulardii* pretreated mice liver might be due to its conversion to spermine and spermidine, the polyamines involved in liver regeneration. Presence of excess of these molecules might have helped in the lowering or prevention of liver damage after administration of CCl_4.

4.4.14. Effect of *S. boulardii* pretreatment on total cholesterol level in CCl_4 induced liver damage in mice

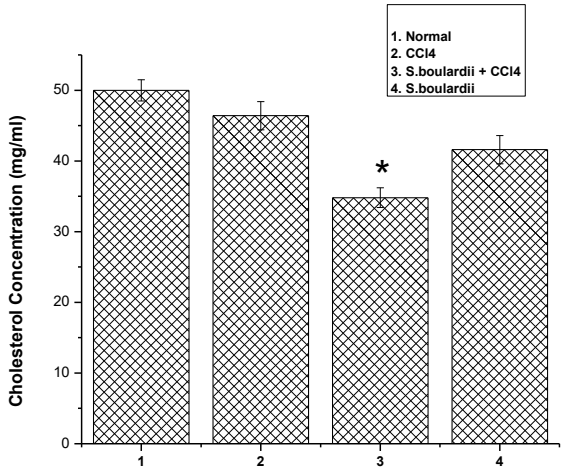


FIGURE 13

Normal Cont: normal control mice
CCl_4 treated: CCl_4 treated mice;
S. boulardii + CCl_4: mice which received *S. boulardii* prior to CCl_4 administration,
S. boulardii: mice received *S. boulardii* alone.

Number of mice in each group was 6 and value in each column represents mean $\pm$ SD. The "*" sign indicates significant increase in total cholesterol concentration in mice treated with *S. boulardii* prior to CCl_4 administration compared to toxin control group.

The above figure (Fig 14) shows the levels of total cholesterol in the serum samples of mice. The total cholesterol concentration was 50 mg/ml in normal mice group. The amount decreased to 41mg/ml in *S. boulardii* treated mice group. The probiotics are known to incorporate cholesterol into their membrane and thus to lower the concentration of cholesterol in host (Ryan et al, 2015.) The value of cholesterol was found to decrease to 47 mg /ml in case of mice treated with CCl_4 and it become 35 mg/ml in case of mice treated with *S. boulardii* prior to CCl_4 treatment.

4.5 DISCUSSION

The current study was undertaken to investigate the role of *S. boulardii* on toxin induced hepatotoxicity *in vivo*. A bioassay model was developed where CCl_4 was used to damage the liver and the role of pretreatment of *S. boulardii* was observed on hepatotoxicity. CCl_4 induces hepatic damage consisting of hepatic steatosis, fibrosis, massive infiltration, centrilobular necrosis and cirrhosis. CCl_4, upon metabolism by NADPH-cytochrome P450 system, is converted to a reactive metabolite trichloromethyl free radical ($\bullet CCl_3$), which may react with O_2 and form trichloromethylperoxy free radical ($Cl_3COO\bullet$) **(**Recknagel et al.1973). This tricholoromethylperoxy free radical hence formed can induce oxidation of the bilayer membrane of different organs along with liver. This damage may result into the leakage of the cellular content into the blood. However, the exact mechanism by which CCl_4 causes liver damage is still unknown and controversial.

ALP and ALT are two enzymes which are found in the intact hepatocytes and are released into the blood stream upon damage of the hepatocytes. Thus, these two enzymes act as markers of liver damage and a higher concentration/activity in serum signifies the liver damage. CCl_4 administration caused elevation of serum ALT and ALP levels in toxin control mice. The levels of these two enzymes were found to decrease in the serum of mice treated with *S. boulardii* prior to CCl_4 administration. This result suggests the potential hepatoprotective role of the yeast against CCl_4 toxicity. The concentration of total protein in serum was found to decrease in mice treated with CCl_4, which is a marker of biosynthetic capacity of liver and decreases during hepatic damage. This hypoproteinaemia may result from decreased protein synthesis by the liver which is a consequence of liver damage. Pretreatment with *S. boulardii* prior to CCl_4 administration showed to prevent the decrease in serum total protein. Moreover, the amount of total protein in the serum of *S. boulardii* pretreated mice was a little higher than the normal amount of serum total protein. It is known that *S. boulardii* activates the adaptive immune response of host and thus induce the production of large amount of gamma globulin. This phenomenon might be be the reason behind this hyperproteinemia in *S. boulardii* treated mice **(**Buts et al, 1990; Nagel et al, 1963).

The hepatoprotective role of *S. boulardii* was further validated by the histological studies of liver sections collected from livers of all the experimental groups. The liver sections of toxin control

mice livers showed considerable amount of necrosis around the central vein whereas less or almost no necrosis was found in the livers of mice treated with *S. boulardii* prior to CCl_4 administration.

The markers of kidney functioning, BUN and creatinine, were measured in the livers of all experimental mice. As urea is secreted by the liver, and removed from the blood by the kidneys, this test is a measure of both liver function as well as renal function. BUN level was found to be elevated in CCl_4 treated mice whereas the level was considerably low in mice treated with *S. boulardii* prior to CCl_4 treatment. As there was no change in the serum creatinine level among all the experimental groups, the change in BUN can be considered as a result of liver function. The dose of CCl_4 used to damage the liver may be was not sufficient to make damages in the kidney as revealed by the histology of kidney taken from the mice treated with CCl_4 or *S. boulardii* showed no prominent damage or recovery.

The major end product of lipid peroxidation i.e MDA, which being highly electrophilic as well as nucleophilic, reacts with primary amino groups of proteins and forms schiff bases, giving rise to intra and intermolecular linkages which can lead to polymerization and inactivation of enzymes **(**Marnett et al, 2002). In addition, reactivity of MDA towards nucleic acid bases of DNA at physiological pH forms adducts resulting in inhibition of transcription and translation. The high concentration of MDA in CCl_4 treated mice indicates the membrane damage in hepatocytes. Treatment with *S. boulardii* prior to CCl_4 administration significantly prevented the damages caused by excessive lipid peroxidation as revealed from lower MDA concentration (Nicholls et al, 1992; Uchida et al, 1990, 93; Stone et al, 1990).

To find out the possible mechanisms of hepatoprotectivity, the amounts of total polyamines were measured in the livers of all experimental groups. It was found that the concentration of total polyamines was increased in damaged liver compared to normal. This observation is correlated with the role of polyamines in the repair process in liver after any damage. The conc. of total polyamine was found to be almost double in the liver of mice received *S. boulardii* only. This might account for the polyamines produced by *S. boulardii* in the gut, which are transported into the liver through the portal vein. The total polyamine conc in the livers of mice received *S. boulardii* prior to toxin was almost at the same level as only *S. boulardii* treated mice liver. This

might account for the feedback inhibition of polyamine synthesis in liver when external sources of polyamines are available (Fleming et al, 2015).

Among the polyamines, putriscine is the precursor polyamine which is converted to spermine and spermidine. Putriscine concentration was found to decrease in the livers of mice treated with *S. boulardii* prior to CCl_4 and the concentrations of spermine and spermidine were increased. This increased value is might be due to de novo SPM synthesis in damaged liver along with *S. boulardii* induced polyamine synthesis as it is already known that SPM concentrations significantly increase in tissues following injury, inflammation, and antigen stimulation, attributed to both its release from dying and injured cells and its stimulated biosynthesis. (Zhang et al, 2000)

The total cholesterol level in the serum of *S. boulardii* treated mice group was found to decrease compared to normal control and CCl_4 treated mice. Probiotics have been found to lower the cholesterol levels and several mechanisms have been proposed for that. One of them is the possible incorporation of cholesterol in the cellular membrane of the yeasts. *S. cerevisiae* and *S. boulardii* can remove cholesterol from laboratory culture media (Jennifer et al, 2015).

4.6 CONCLUSION

- The probiotic yeast *S. boulardii* possesses protective activity against CCl_4 induced hepatotoxicity in mouse. Pretreatment with *S. boulardii* prior to CCl_4 intoxication was found to prevent the elevation of serum GPT, ALP and other markers.

- The concentrations of polyamines increase in liver in *S. boulardii* treatment mice and might be one of the reasons behind hepatoprotective role of *S. boulardii* as spermidine induces macrophage mediated autophagy in gut and liver. It can be hypothesized that the polyamines produced by *S. boulardii* in the gut are transported to the liver through the portal vein and increase the polyamine concentration in there leading to liver regeneration.

- It is too early to comment on the mechanism of action of the hepatoprotectivity and more studies must be conducted to elucidate the role of the *S. boulardii* in this aspect.

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OVERALL SUMMARY

- The growth of *S. boulardii* varied with the nature of the prebiotic and inulin acted as a better prebiotic for the yeast.
- The total number of microorganisms reduced in the fecal matter when mice were treated with the synbiotic combination of *S. boulardii* and inulin, *S. boulardii* or inulin alone.
- *In vitro* association studies showed aggregate formation between *E. coli/E. faecalis* and *S. boulardii* in presence of inulin within 24 hrs. Aggregate formation did not observed between *S. boulardii* and other microorganisms like *S. aureus* and lactobacilli. The possible reason of *in vivo* reduction in number of microorganisms might be the aggregate formation between *E. coli/E. faecalis* and *S. boulardii* in presence of inulin and subsequent elimination from the gut.
- The aggregate formation is a property of live *S. boulardii* as heat treatment abolished aggregation and surface proteins of *S. boulardii* might be the involvement in the interaction as cycloheximide treatment reduced the extent of aggregation.
- The aggregation between *E. coli/E. faecalis* and *S. boulardii* was not found in presence of other prebiotics like fructooligosaccharide or maltodextrin and aggregate formation increased with time in presence of inulin.
- The antibiotic sensitivity of lactobacilli varies significantly with prebiotics. *L. casei* was found to become highly sensitive to azithromycin in presence of maltodextrin compared to *L. plantarum* and *L. rhamnosus.* MTT assay showed increased cell viability of *L. casei* and SEM analysis revealed considerable reduction in size of the bacteria. Similar observation was seen in case of *L. plantarum* which showed increased sensitivity to azithromycin when grown in inulin. Cell viability assay and fluorescence microscopic pictures revealed less viability and perforated membrane structure for *L. plantarum* while *L. rhamnosus* membrane remained intact in presence of inulin.
- Hence, choice of a prebiotic and probiotic for better synbiotic effect is very important for any treatment and particularly along with antibiotics.
- Pretreatment of mice with *S. boulardii* prevented CCl_4 induced liver damage.
- Increase in polyamine concentration in liver in presence of *S. boulardii* might be one of the reasons that protected the liver and other physiological parameters.

List of Publications

Ganguly R[1], **Chakraborty R**[2], Sarkar, K. Inulin induced co-aggregation of Sacharomyces boulardii with potential pathogenic bacteria. *International Journal of Probiotics and Prebiotics*; 2019; 14: 18–23. [1 and 2 contributed equally]

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Sarkar D, Mal P, Sinha S, **Chakraborty R** and Sarkar K. Prevention of carbon tetrachloride induced hepatic damage in mice by the probiotic yeast *Saccharomyces boulardii*. *International Journal of Biology, Pharmacy and Allied Science;*2013; 2(4): 879-893.