PROPHYLACTIC ROLE OF *BACILLUS CLAUSII* AGAINST ARSENIC-INDUCED LIVER DAMAGE IN RAT

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Certificate of Approval

This is to certify that thesis entitled "**Prophylactic Role of *Bacillus clausii* Against Arsenic-Induced Liver Damage In Rat**" has been carried out by Ms. Shrestha Bhanja under the supervision of Prof. Saikat Dewanjee, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032. She has incorporated her findings into this thesis of the same title, being submitted by her, in partial fulfillment of the requirements for the degree of "Master of Pharmacy" of this university. She has pursued this work independently with proper attention to my entire satisfaction.

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DECLARATION

I, the undersigned solemnly declare that the project report on "**Prophylactic Role of *Bacillus clausii* Against Arsenic-Induced Liver Damage In Rat**" is submitted in the partial fulfillment of the degree of "Master of Pharmacy", under the supervision of Prof. Saikat Dewanjee, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032. I assert the statements made and conclusions drawn are an outcome of my research work. I further certify that the work contained in the report is original and has been done by me under the general supervision of my supervisor.

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DEDICATED TO MY PARENTS

Mr. Sanjay Kumar bhanja and Mrs. Kakali Bhanja

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PREFACE

Arsenic is one of the most threatening pollutants found excessively in the groundwater. Thus, contributing to drinking water contamination. Daily intake of this polluted drinking water causes induction of oxidative stress pathway mediated cell damage in humans and animals. The main treatment for arsenic poisoning is to eliminate arsenic from the tissues of the body. Chelators have showed some promise in the treatment of acute arsenic poisoning, but it is unclear whether they will be as successful in treating chronic instances. Henceforth, New strategies are required because the majority of chelators have drawbacks and negative effects. There are difficulties using phytochemicals to reduce arsenic toxicity. Probiotics have been found to improve oxidative stress, inflammation, and fibrosis in several liver conditions. They accomplish this by altering the typical gut bacteria's makeup and functionality, which supports a healthy digestive tract. As a result, energy production is enhanced and harmful liver consequences are avoided. Additionally, probiotics lessen liver oxidative stress by triggering the body's antioxidant system. Additionally, certain probiotics trigger signals that boost the synthesis of antioxidant compounds and enzymes. Probiotics also have a metal-chelating impact that improves their antioxidant activity. Hence, probiotics may evolve as potential next generation alternative treatment for arsenic induced hepatotoxicity. In order to cure liver damage brought on by arsenic and provide better and safer treatment results, *Bacillus clausii* will be a good choice.

This thesis has been organized into six distinct chapters. Chapter 1 provides a concise introduction to the mechanisms of arsenic-mediated hepatotoxicity and highlights the limitations of conventional therapeutic strategies. Chapter 2 presents a comprehensive literature survey on *Bacillus clausii* in its relevant context. Chapter 3 outlines the materials and methods employed to conduct the research. Chapter 4 focuses on the results and includes relevant discussions. Chapter 5 presents the conclusion of the study, while chapter 6 lists the references that contributed to the significant findings of this research

Chapter 1

Introduction

Contents

1. Introduction

The environment contains naturally occurring arsenic. It has ample presence in the earth and groundwater. However, exposure to excessive quantities of the element can be harmful. Numerous forms of health hazards can be brought on by prolonged exposure (Carlin et al., 2016). Chronic arsenic exposure from polluted water sources is an issue for world health. On the other hand, contamination rates are significantly influenced by regional dispersion. Less than 10 ppb of arsenic are considered safe for drinking according to the World Health Organisation (Yunus et al., 2016). Personal reservoirs across the world, including in the US, have produced contaminated drinking water. Exposure happens at work when making pesticides, herbicides, glass, semiconductors, smelting, mining, or doing tasks like carpentry that entail burning, removing, or being around buildings and/or materials that have been treated with arsenate wood preservatives (Hughes MF, 2002). There are primarily three kinds of arsenic: organic, inorganic, and arsine gas. There are also three main valence states for arsenic: elemental arsenic (valency 0), trivalent arsenite (valency +3), and pentavalent arsenate (valency +5). Pentavalent substances are generally thought to be less dangerous than trivalent compounds, which include both inorganic and organic. In general, organic arsenic is thought to be less harmful than inorganic arsenic (Kuivenhoven and Mason, 2023). Arsenic exerts a variety of impacts on the blood biochemistry, enzymes, and function of transport proteins (Markovich and James, 1999; Dash et al., 2007). Excessive production of reactive oxygen species (ROS) may incapacitate the body's antioxidant defence leading to radical deterioration of DNA, lipids, and proteins (Ramírez et al. 2000). Toxin and carcinogen removal is mostly carried out by the liver's metabolic activity. Acute hepatitis and cholestasis are possible symptoms of arsenic induced liver damage, which might also progress to liver cirrhosis. The buildup of lipid-derived oxidation products that damage the liver and result in cell necrosis is facilitated by ROS produced by metabolic intermediates of xenobiotics through stimulation of CYP450 families as well as by activated inflammatory cells through NADPH oxidases (Singh et al.2014).

1.1. Hepatotoxicity

Hepatotoxicity refers to the ability of certain substances, medications, or conditions to cause damage or injury to the liver. The liver is a vital organ

responsible for various essential functions in the body, such as metabolism, detoxification, and synthesis of proteins. When exposed to hepatotoxic agents or factors, the liver's normal structure and function may be compromised, leading to various liver-related issues. Hepatic damage can be hepatocellular, cholestatic or mixed type (Andrade et al., 2005). Cholestatic damage is frequently caused by the medication or its metabolite. They block the hepatobiliary transporter system which are necessary for the production of bile and the release of xenobiotics and cholephilic compounds (Pauli-Magnus and Meier, 2006). Nonsteroidal anti-inflammatory medications (NSAIDs), anti-infectives (anti-tubercular drugs), anti-cancer therapies, hormone drugs, immunosuppressive agents, sedatives, and neuropsychiatric pharmaceuticals are among the agents leading to drug-induced liver impairment (Yu et al., 2017). Acetaminophen is most frequently linked to drug-induced liver damage (Holubek et al. 2006). If we consider the histopathology of cholestatic hepatic damage, it can be of two types chronic and acute. In acute cholestasis, bile plugging is observed. Whereas, in chronic type, portal inflammation, bile injury, bile stasis, duct paucity is seen (Fontana, 2014, Real et al., 2019). Hepatocellular damage can form through multiple pathways including direct hepatotoxicity by toxic agents and immune response system of the body (Ye et al., 2018). Hepatotoxic substances can be metals; like arsenic, beryllium, iron, copper, and manganese (Renu et al., 2021). Endogenous compounds such as androgens, estrogens, glucocorticoids and vitamin A can cause hepatocellular damage in the liver (Xu et al., 2022). Apart from these, xenobiotics; like allyl alcohol, carbon tetrachloride, hexachlorobenzene, vinyl chloride etc. can also lead to hepatotoxicity (Sturgill and Lambert, 1997). Some toxins are also responsible for hepatic damage, these toxins can be mycotoxins (aflatoxin B1, fumonisins, sporidesmin, amatoxin, phalloidin) or plant toxins (pyrrolizidine alkaloids, lantadene) (Quan et al., 2020; Hua et al., 2021). Acute hepatotoxicity is one of the hepatocellular damage, which is defined by severe inflammation with necrosis and apoptosis in liver tissues and another one is chronic type which is distinguished from acute one by formation of tissue fibrosis (Kleiner et al., 2013). Acute or chronic liver disease can be distinguished by evaluation of liver enzyme levels (G, 1998). Increased serum alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) levels often represent the severity of decreased bile flow or

cholestasis, whereas increased serum alanine (ALT) and aspartate (AST) aminotransferase levels typically reflect the severity of hepatocellular damage (Iluz-Freundlich et al., 2019). Other forms of liver damage is characterised by microvesicular and mitrochondrial damage, as seen in steatosis; zonal necrosis is another type which is usually associated with drug induced liver toxicity. Granulomas are also associated with hepatotoxicity (Navarro et al., 2017).

1.1.1. Agents causing hepatic damage

Numerous potential agents, both endogenous and exogenous can lead to hepatotoxicity. Among the indigenous agents; immune system dysfunctionality, genetic liver disorder are some of the important factors. Other factors, such as androgens, oestrogens and vitamin A overdose can also be potent cause of hepatocellular damage. On the other side; drugs, xenobiotics and environmental pollutants are the major concerns among the exogenous factors of hepatotoxicity. Over exposure to exceeded amounts of metals like lead, chromium, arsenic, mercury, nickel and cadmium triggers hepatic damage by production of ROS, which is responsible to bring about different unfavourable alterations in the hepatocytes (Renu et. al. 2021). Basic biological functions like growth, proliferation, differentiation and apoptosis are all impacted by heavy metals toxicity (Balali-Mood et al., 2021). ROS are overproduced in hepatocytes as a result of oxidative stress caused by heavy metals, which causes radicals to bind covalently to macromolecular proteins or lipids found in the membranes of cellular organelles (Teschke, 2022). Hepatotoxic effects of heavy metals have also been associated with inflammation including TNF-alpha, inflammatory cytokines, MAPK, and ERK pathways (Renu et al., 2021).

1.1.2. Arsenic: a potential pollutant to cause hepatotoxicity

Arsenic is a hazardous metalloid, much responsible for heightening the health concerns of humans and animals (Das et al. 2018). It is predominantly found in the environment as inorganic arsenite. Whereas, arsine and organic arsenic are other forms (Balali-Mood et al. 2021). Experimentally arsenic was found to possess cytotoxic effect in hepatocytes through formation of certain free radicals and ROS (Teschke, 2022). Nitric oxide, singlet oxygen, hydrogen peroxide, peroxyl radical, dimethyl arsinic radical, and dimethyl arsinic peroxyl radical are specifically mentioned among these (Briffa et al. 2020). Due to the rapid

processes that prevent immediate capture, the chemical mechanisms that lead to these radicals are challenging to firmly establish. (Balali-Mood et al. 2021) The metabolism of arsenicals occurs mostly in the liver, which is a target organ both for toxicity and metabolism (Balali-Mood et al., 2021, Islam et al., 2011). This metabolism produces the methylated intermediates monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Islam et al., 2011). Arsenite methyltransferase and DMA methyltransferase, two enzymes found in the cytosol of hepatocytes, are responsible for the synthesis of MMA and DMA, respectively, in the metabolism of arsenicals (Teschke, 2022). Arsenic impact on oxidative stress by increasing the levels of ROS, MDA and NO and at the same time, lowering antioxidant levels including reduced GSH, catalase, and SOD. Apoptosis (AKT-PKB, MAPK, PI3/AKT, PKCδ-JNK, AKT/ERK, and p53 pathways), fibrosis (TGF-β/Smad pathway), necrosis, and inflammation (TNF-α, NF-κB, IL-1, and IL-6) are among ways that oxidative stress causes mitochondrial dysfunction (Renu K et al., 2021). Pro-inflammatory cytokines shows altered expression levels as a result of arsenic intoxication. (Liu et al., 2021). Further, arsenic triggers the apoptosis regulatory system, activates caspases and Bax, reduces Bcl2 due to mitochondrial malfunction, and activates caspases and Bax. Researchers presume arsenic-induced hepatotoxicity is caused by the modification of all these mechanisms (Renu K et al., 2021).

1.1.3. Available traditional treatments for liver disease

Conventional treatment for hepatic disease is mostly lifestyle modification and in extreme cases liver transplant. Thus, majority of people trusts on traditional herbal healing methods for liver diseases. *Silymarin*, *Glycyrrhizin*, *Phyllanthu*s are some of the herbs, useful in reversing hepatocellular damage (Stickel and Schuppan, 2007). A combination of flavonolignans known as silymarin was discovered in milk thistle seeds called Silybum marianum (L.) silibinin, isosilibinin, silicristin, and silidianin are some of the main components of silymarin. Globally renowned herbal treatment for chronic liver problems is silymarin (Kidd and Head, 2005). The main active ingredient of silymarin, which has been extensively researched for its hepatoprotective properties, is silibinin (Kidd and Head, 2005). In China and Japan, hepatitis folk medicine has traditionally employed glycyrrhizin, an active ingredient in Glycyrrhiza uralensis Fisch. Since 1948, Japan has allowed

the use of glycyrrhizin injection for the treatment of allergic inflammation, and since 1979, chronic hepatitis (Kumada, 2002). Significant anti-inflammatory effects of glycyrrhizin have been identified in the liver and other tissues. The processes may involve engaging both conventional and non-conventional pathways to inhibit complement's cytolytic activity. Further research revealed that glycyrrhizin inhibits the lytic process that results in the formation of the membrane attack complex (Fujisawa et al., 2000). A subtropical plant called *Phyllanthus niruri* L, which is extensively distributed in China and South Asia, has been utilised in traditional Chinese medicine to treat chronic hepatitis. Phyllanthus niruri L's major active ingredients, which include quercetin rhamnoside, gallic acid, geraniin, and quercetin glucoside, have demonstrated anti-hepatotoxic characteristics (Liu et al., 2014, Ibrahim et al., 2013, Manjrekar et al., 2008, Amin et al., 2012). Some examples of traditional plants exhibiting hepatoprotective action is listed in **Table 1.**

Table 1. Examples of some traditional hepatoprotective plants

Botanical names	Parts used	Hepatotoxicity inducing agents	References
Amaranthus caudatus Linn	Whole plant	CCl_4	Ashok et al., 2011
Asparagus racemosus Linn	Roots	Paracetamol	Rahiman et al., 2011
Cajanus cajan Linn	Pigeon pea leaf	D-galactosamine	Akinloye et al., 2011
Ficus religiosa Linn	Stem bark	Paracetamol	Suryawanshi et al., 2011
Garcinia indica Linn	Fruit rind	CCl_4	Deore et al., 2011
Solanum nigram Linn	Fruits	CCl_4	Subash et. al., 2011

1.2. Probiotics

The liver-gut axis, as it is known, has a strong connection to the digestive system. The liver's actions are influenced by the intestinal blood, which in turn feeds the portal system. However, the liver regulates the function of the intestines and secretes bile (Zeuzem, 2000). A comprehensive ecological system is formed by intestinal microorganisms. More than 500 species of microorganisms make up the microbial ecosystem of the gut, the majority of which are still not cultured and many of which are yet unexplored. There are two types of bacteria which

contributes to the intestinal flora, they are either fixed in the gut (autochthonous, resident) or bacteria that just travel through the intestine (transient allochthonous) form part of the intestinal microflora (Saavedra and Tschernia, 2002). Numerous significant physiological processes are carried out by the gut microbiota. It creates vitamins, breaks down bile acids, breaks down minerals, and provides a crucial barrier against infections by creating both local and systemic immunity (Abt and Artis, 2009). It is well-accepted that individuals with liver cirrhosis experience aberrant colonic bacterial proliferation in their small intestine. Intestinal bacterial overgrowth affects at least 50% of patients. In contrast, healthy people have very little of such bacteria (Lo et al. 2014). Probiotics are living things or compounds that benefit the host animal by restoring the balance of the gut flora. They are non infectious living microorganisms, such as certain beneficial bacteria, that, when given to the host in an appropriate amount, impart health-promoting as well as disease-preventing qualities (Dewanjee et al. 2022). Probiotics' role in regulating immune, respiratory, and gastrointestinal systems is now extensively understood and subject to scientific analysis (Floch et al. 2011). They have been demonstrated to perform a protective effect by directly competing against intestinal pathogens through the generation of metabolites like acetic acid and lactic acid as well as antibacterial compounds like bacteriocins (Cotter et al. 2005; Servin AL, 2004). Research on the interactions between beneficial bacteria (also known as the microbiota), pathogens, and the human body may lead to new developments in probiotics, even if the majority of investigations have been empirical. It is crucial to comprehend the biological processes of gut colonisation in both healthy and inflammatory states when creating probiotics for particular uses (Behnsen et al., 2013).

1.2.1. Types of probiotics

Probiotics generally work in the gastrointestinal system, where they might alter the intestinal flora. Depending on the background microbiota, probiotic strain, and gastrointestinal tract area, probiotics can infrequently colonise the human gut epithelium in highly specific ways (Zmora et al., 2018). Probiotic effects can vary depending on the probiotic species and strains used, therefore precise guidelines for their usage in clinical trials and other research are required. Therefore, combining information from research on many probiotic varieties might lead to

inaccurate conclusions regarding the efficacy and security of those probiotics (Sanders, 2015). Probiotics are recognised by their unique strain, which contains the genus, species, subspecies (if appropriate), and an alphanumeric strain name. The seven main bacteria genera that are most frequently found in probiotic products are Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus, Escherichia, and Bacillus. There are many different strains and dosages of probiotics available as dietary supplements (in capsules, powders, liquids, and other forms) (Szajewska et al., 2016). Instead of just one strain, these items frequently contain mixed cultures of living microorganisms. It is challenging for people who are not familiar with probiotic research to understand which products are supported by evidence because the effects of many commercial goods containing "probiotics" have not been investigated in research studies. Nevertheless, several organisations have thoroughly examined the existing data and produced recommendations on certain probiotics, including the best brand, dosage, and formulation, to use for preventing or treating a range of illnesses (Hill et al., 2014).

1.2.2. Therapeutic roles of probiotics

Probiotics generally act by three possible way (Hill et al., 2014). Generalised or non-specific pathway differ significantly amongst probiotic supplement strains, species, and even taxa. These mechanisms include the production of bioactive metabolites (such as short-chain fatty acids), the reduction of luminal pH in the colon and the inhibition of the growth of pathogenic microorganisms in the gastrointestinal tract (by encouraging colonisation resistance, enhancing intestinal transit, or aiding in the normalisation of a disturbed microbiota). The second type is species specific, where the mechanism is bile salt metabolism, enzymatic activity, gut barrier reinforcement, vitamin synthesis and toxin neutralisation. Another type of mechanism of probiotics is strain specific, which is uncommon and only used by few strains. Cytokine generation, immunomodulation, and impacts on the endocrine and neurological systems are a few examples of the processes adopted by these strains (Sanders, 2015). A single probiotic variant or a blend of variants have previously been proven in multiple experiments in mice as well as humans to modify gut function and treat illness. Numerous of these studies have shown encouraging findings about the use of probiotics in the management

of acute gastroenteritis, diarrhoea or colitis caused by *Clostridium difficile*, inflammatory bowel syndrome, necrotizing colitis, and other conditions (Preidis and Versalovic, 2009).

1.2.3 Probiotics as therapeutic tool against hepatotoxicity

Alteration of gut microbiome has importance in healing liver tissue damage. Thus, there is an inevitable role of probiotics in hepatoprotection. With proper dose maintenance probiotics can contribute to therapeutics of liver injury (Dewanjee et al., 2022). There is a noticeable progress in the study of probiotic drugs for different liver diseases over the past decade. Though some more research and clinical data is needed for the establishment of the facts, it can be guaranteed that probiotics are an answer for the treatment of metabolic associated fatty liver disease (MAFLD), alcoholic liver disease, primary sclerosing cholangitis and autoimmune hepatitis. Recently, it has been found that imbalance of gut microbiota is one of the most important pathophysiology of primary cholangitis, Wilson's disease, hepatitis B and hepatitis C. Probiotics possess the potential to rectify the gut microbial disbalance which establishes its importance as alternative therapy (Maslennikov et al., 2021). Probiotics have been beneficial for serum liver enzyme concentrations, liver steatosis, lipid profiles, and liver stiffness, according to earlier investigations (Khalesi et al., 2018; Liu et al., 2019). Furthermore, it has been discovered that supplements containing *Lactobacillus sp*. or *Clostridium butyricum* assist in reducing the acute liver injury triggered due to CCl_4 (Chen et al., 2018; Liu et al., 2017). *Lactobacillus rhamnosus* is found to increase transcription factor Nrf2 in liver that is responsible for defence against xenobiotics and oxidative stress caused by acetaminophen (Saeedi et al., 2020). The blend of *Bacillus sp* spores in MegaSporeBioticTM probiotic capsules ameliorate histopathological injury to the liver and reduce the level of proinflammatory cytokines, demonstrating that they have a beneficial impact on acute hepatic injury brought on by acetaminophen (Neag et al., 2020). All these informations are crucial for the therapeutic management of acute liver injuries. Thus, further investigations must be carried out to ensure if probiotic supplementation might enhance the capacity of hepatocytes to antioxidant defence and detoxification (Chen et al., 2021).

1.3. Origin of problem

Arsenic is widely available major pollutant of drinking water in many countries like India, Nepal, Pakisthan, Afganisthan, China, Bangladesh, Mexico, Argentina, Africa and many others. If the permissible amount of daily arsenic consumption is exceeded, it may lead to several health concerns. Arsenic mostly affects the major organs like kidney, liver, heart, lungs brain and blood. Thus, toxicity caused by arsenic is a matter of global discussion in present era. Despite being such a major issue, there is no defined treatment strategy for arsenic intoxication related health issues. The primary treatment strategy is chelation by 2,3-dimercaprol, meso-2,3-dimercaptosuccinic acid and 2,3 dimercaptopropane-1-sulfonate. However, this therapy involves limitation such as removal of essential metal ions along with arsenic. As arsenic majorly affects organ tissues by oxidative stress pathway, liver tissues are one of the most damaged tissues. Hepatotoxicity is currently difficult to treat with conventional medicine. Due to its multifaceted approach, alternative herbal therapy offers more drawbacks than benefits for treating a particular condition. The herbal extracts act on toxicity affected hepatocytes but along with this they show effects on numerous receptor and produces thousands of activity which may be unwanted. In this scenario, a new treatment strategy is needed to combat the arsenic induced hepatotoxicity. Probiotics have the potential to evolve as an answer for this search. It is already established that they possess a role in altering the gut microbiome system and generating a beneficial ecosystem in the gut. A healthy gut can be responsible for many immunotherapeutic role, antioxidant effect and many more. Further scientific exploration is needed for probiotics to understand its full potential. Thus, different strains of probiotics are needed to be investigated properly to establish their effect against hepatotoxicity.

1.4. Objective

The principal objective of the research is to explore the therapeutic potential of the probiotic supplement in arsenic-induced hepatotoxicity in vivo. In addition, efforts would be made to discover the protective mechanism of probiotics and their therapeutic effect.

Chapter 2

Literature Reviews

Contents

2. Literature reviews

Detailed Literature survey is one of the most important requisite before performing research work. Before finalizing the probiotic strain used for this in vivo study, an in depth relevant literature survey was done. *Bacillus clausii* was selected as experimental probiotic considering its diverse potential. Furthermore, *B. clausii* exhibits excellent gastric acid tolerance making it suitable for oral administration. The increasing number of reseach work in the past decade involving this probiotic signals exploration of more potential.

2.1. Role of *B. clausii* in gastro-intestinal protection

The misuse of antibiotics can drastically change the microbiota, resulting in imbalance and instability of the gastrointestinal mucosa. *Bacillus clausii* spores were found to reduce the proinflammatory cytokines like IL-1β and TNF-α and increase the anti-inflammatory type cytokines IL -10 in colonic inflammation. Additionally, *B. clausii* checks elevated ALT, AST and LDH level as found in ELISA assay (Pirozzi et al., 2023). When compared to placebo, the spores of B. clausii mildly improves acute colitis. Although this benefit was shown during the recovery phase of colitis, when the clinical symptoms were nearly discrete in both control and treated mice, *B. clausii* appears to improve the whole histopathology of colitic mice when administered at a lower dose (Scaldaferri et al., 2021). B. clausii spores aids in attenuating the extremity of diarrhoea in children under 5 years of age. The number of patients recovered was more in the group taking probiotic B. clausii supplement., most effective after 72 h treatment. Number and frequency diarrhoea episodes were reduced after this treatment (Sudha et al., 2019).

2.2. Role of *B. clausii* in strengthening immunity

In a human enterocyte model of rotavirus infection, the *B. clausii* probiotic strain was studied to have protective benefits and numerous non-immune mucosal barrier and innate immune system defence mechanisms. Up-regulation of TLR3, NF-κB1, MyD88 and TRAF6 expression mediated by rotavirus can be controlled by *B. clausii* administration (Paparo et al., 2020). *B. clausii* exhibits an immunomodulatory influence in *E. coli*-infected mice by regulating lymphoid hyperplasia in the spleen and mesenteric lymph nodes, increasing mucin production, and reducing mucosal depletion in the small intestine (Yu et al., 2016).

Rats infected with *Staphylococcus aureus* had significantly higher levels of all liver enzymes ALT, AST, and ALP, as well as higher quantities of creatinine and urea. Oral treatment with probiotic *B. clausii* was found to reduce the liver tissue damage, decrease the liver enzyme leakage in the plasma. It also caused slight reduction in urea level and significant attenuation of creatinine level, which implies improvement in kidney function (Werdi et al., 2023). A randomized, double blind controlled study was performed to validate the potential of *B. clausii* capsules in minimizing the adverse effects of *Helicobacter pylori* eradication therapy. It was concluded that administration of *B. clausii* during the H. pylori eradication therapy significantly reduced the incidence of diarrhea associated with the treatment (Plomer et al., 2020).

2.3. Role of *B. clausii* in renal protection

The probiotic strain *B.clausii* exhibits positive impact on renal oxidative stress in lipopolysaccharide (LPS) induced endotoxemia. It was observed that it significantly reduces Total Antioxidant Status (TOS), Oxidative Stress Index (OSI) in renal tissues and increases Total Antioxidant Status (TAS) level. Additionally, it reduces malonaldehyde (MDA), myeloperoxidase (MPO). When compared to the LPS group, the probiotic therapy diminished IL-6 and TNF-α response (Kandil A, 2021).

2.4. Role of *B. clausii* regarding asthmatic response

Probiotics may encourage reactions that are anti-inflammatory and anti-allergic. By modifying the Th2 pathway, which is mediated by HIF1 signaling in the mouse model, *Bacillus clausii* significantly contributes to the suppression of asthmatic responses in OVA-induced mice. It decreases the Ig E, IL4, IL5 levels which are related to asthma (Park et al., 2020).

2.5. Role of *B. clausii* in hepatoprotection

Liver inflammation and endotoxemia are caused by increased levels of oxidative stress indicators (ROS, AOPP, TBARS, TOS, and OSI) and liver tissue inflammation (MPO) following LPS administration. *Bacillus clausii* promotes antioxidant levels to maintain cell viability. It protects the hepatocytes by increasing CAT and SOD antioxidant enzyme activities in rats. TAS and GSH levels were also significantly increased by this supplementation (Sougat et al., 2022). Histopathological evidence has been found for establishment of the fact

that *Bacillus clausii* protects the liver tissues against the toxic effects of aflatoxin B1 in rat model. The aforementioned probiotics possesses antifungal activity, thus reducing the sustainability of pathogenic fungi *Aspergillus flavus*, known for producing the aflatoxin B1. When compared to Bacillus subtilis for antifungal activity, *B. clausii* shows to be more potent (AL-Masari and Al-Obaidi, 2022). Combination therapy of *Lactobacillus plantarum* and *Bacillus clausii* shows beneficiary action in acute hepatic encephalopathy induced by thioacetamide. An experiment was performed in rat model to evaluate the serum AST, ALP, ALT and ammonia level along with oxidative stress parameter and behavioural parameters. This probiotic combination therapy exerted hepatoprotective and neuroprotective activity. In behavioural parameter tests, i.e., rotarod test, T maze test and object recognition test the group receiving probiotic treatment shows positive result. Escalation of liver enzymes like ALT, AST and ALP in serum was controlled in treated rats which implies less damage of liver tissues. Furthermore, Probiotics inhibited the ammonia released from damaged liver cells from being ingested into the small intestine (Shahgond et al., 2022).

Chapter 3

Materials and Methods

Contents

3. Materials and methods

3.1. Chemicals

Bovine serum albumin (BSA), Bradford reagent, glycerol, hydrogen peroxide solution, hydrochloric acid solution, sodium chloride, butanol, sodium azide, and pyridine were obtained from Sigma-Aldrich, MO, USA. Kits for the measurement of different biochemical parameters were purchased from ARKRAY Healthcare Private Limited, India. Kits for pro-inflammatory cytokinin assay were acquired from Raybiotech, Georgia, USA. *Bacillus* MRS broth and tryptone soya broth were purchased from Himedia Laboratories Private Limited, India. Sodium arsenite ($NaAsO_2$), ethylenediaminetetraacetic acid (EDTA), tris HCl, potassium dihydrogen phosphate, disodium hydrogen phosphate, 5,5'-dithiobis-(2-nitrobenzoic acid), methanol, nitro blue tetrazolium (NBT), NADH, phenazine methosulphate, GSH, 2,4-Dinitrophenylhydrazine, trichloroacetic acid, ethanol, dichlorodihydrofluorescein diacetate (DCFDA), sodium dodecyl sulfate, and thiobarbituric acid were obtained from Sisco Research Laboratory, Mumbai, India. All other reagents used were of analytical grade.

3.2. Preparation of bacterial strain

The *Bacillus clausii* UBBC07 used in this study was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) at the Institute of Microbial Technology (IMTECH) in Chandigarh, India. The frozen stock culture of this strain was grown on De Man, Rogosa and Sharpe agar (MRS) media for 72 hours at 37˚C. A single colony was then transferred to MRS broth and grown at 37˚C for 24 hours. Following the transfer, cells were inoculated in 250 ml of MRS broth and grown for 24 hours. The cells were harvested by centrifugation and aliquots of 10^8 colony forming units (CFU) were prepared in trypticase soy broth with 10% glycerol and stored at -80˚C. Before administration, the cells were washed twice in sterile phosphate buffer saline (PBS) and resuspended in 1 ml of PBS. Each rat was then orally administered with 1 ml of PBS containing 10^8 CFU of this probiotic bacteria strain.

3.3. Animals

Twenty four male Wistar rats (weighing 150-170 g) were obtained from Saha Enterprise, Kolkata, India. They were then placed in standard polypropylene cages measuring 29 × 22 × 14 cm and housed in the animal house of the Department of

Pharmaceutical Technology at Jadavpur University, Kolkata, India. The rats were carefully provided with optimal temperature and humidity conditions, and a balanced 12 hour cycle of light and darkness (Dewanjee et al., 2013). The rats were fed standard diet and water ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee, Department of Pharmaceutical Technology, Jadavpur University (approval number JU/IAEC-22/35). Experimental procedures were carried out according to the principles of laboratory animal care (Public Health Service, 2015). The animals were given a period of 2 weeks to acclimatize before the in vivo experiment was conducted.

3.4. In vivo bioassay

3.4.1. Experimental set up

The in vivo experiment was performed according to the established protocol in Advanced Pharmacognosy Research laboratory, Jadavpur University, India (Das et al., 2010, Dua et al., 2016). Twenty-four male Wistar rats were divided into four groups (n=6) and treated as follows:

Gr I: Control group: Rats were fed with phosphate buffer saline (PBS) (1.0 ml) via an oral gavage once a day for 10 days.

Gr II: Toxic control group: Rats were treated with $NaAsO_2$ (10 mg/kg body weight, p.o., once daily) via an oral gavage for 10 days.

Gr III: Only Treatment group: 1 ml of *B. clausii* (1×10^8 CFU/ml) was orally administered in the experimental rats once daily for a period of 10 days.

Gr IV: Toxin + Treatment group: Rats received a daily dose of $NaAsO_2$ at a concentration of 10 mg/kg body weight, along with 1 ml of *B. clausii* (at a concentration of 1×10^8 CFU/ml) administered orally through gavage, once a day for a period of 10 days.

Based on a thorough literature search that evaluated the numerous therapeutic effects this probiotic strain has shown, the dose of *B. clausii* was selected.

The daily food and water intake was monitored. At the end of the experiment, rats were fasted overnight for 16 h, and on the 11th day, they were sacrificed under euthanasia. The blood samples were collected from retro-orbital venous plexus. The serum was carefully obtained through a gentle centrifugation process, with a rotation speed of 3,000 rpm for 10 minutes. The livers were removed and cleaned using PBS. The weight of each liver was recorded. One portion of liver tissue was

preserved in 10% formalin for histological examinations, while the other portion was immediately homogenized in Tris-HCl (0.01 M) + EDTA (0.001 M) buffers with a pH of 7.4. The tissue homogenate was obtained by centrifuging it at 4°C for 30 minutes at 12,000 x g. The collected supernatant was then utilized for the experiments. Samples of urine were collected from the bladder and then promptly stored at a temperature of -80°C (Khanra et al., 2017). A schematic overview of the in vivo assay has been presented in **Figure 1**.

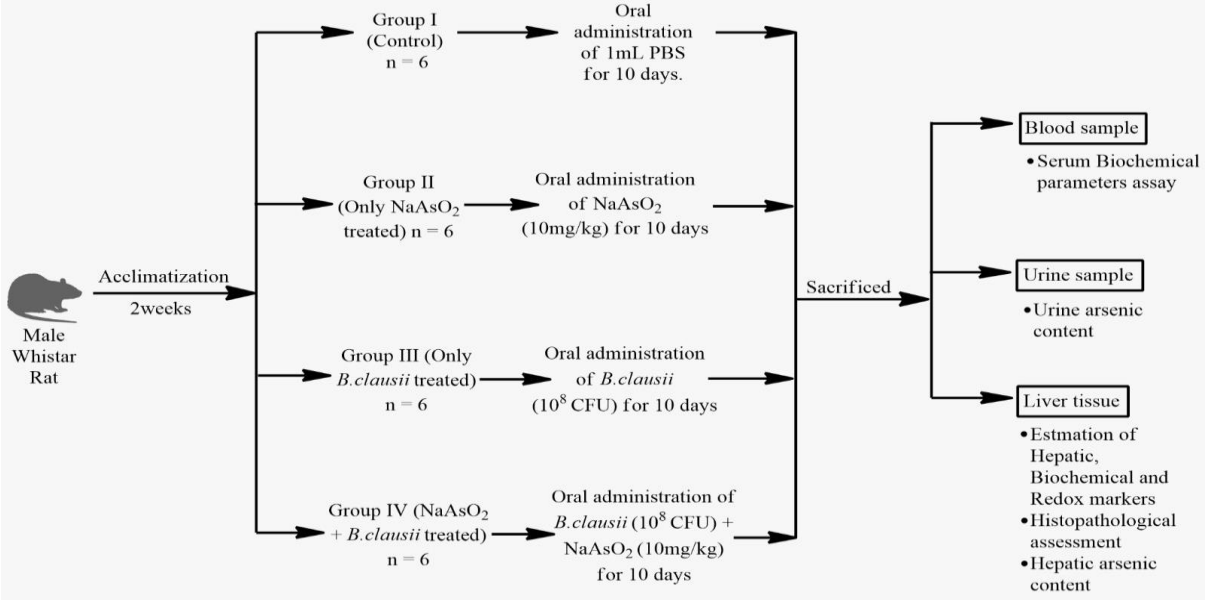

Figure 1. Schematic overview of the experimental protocol

3.4.2. Estimation of Serum Biochemical Parameters

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatinine kinase (CK), and C-reactive protein (CRP) in the blood serum were determined using commercially available kits (ARKRAY Healthcare Private Limited, India) according to the manufacturer's protocol.

3.4.3. Evaluation of serum lipid profile

Serum levels of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were measured according to the instructions provided by the manufacturer of commercially available enzymatic kits (purchased from ARKRAY Healthcare Private Limited, India). Friedewald's formula was used to calculate the low-density lipoprotein (LDL-C) levels in blood serum.

3.4.4. Assays for hepatic and urinary arsenic

An atomic absorption spectrophotometer (Perkin Elmer model number 3100, USA) was used to analyse the levels of arsenic in the liver and urine of test animals in accordance with a predetermined methodology (Das et al., 1995).

3.4.5. Assessment of biochemical and redox markers in liver

The hepatic tissue of the experimental wistar rats were subjected to evaluation for the levels of ROS, GSH, SOD, CAT, lipid peroxidation, and protein carbonylation. These parameters were measured in the hepatic tissue of the rats according to established protocols after receiving various treatments. (Manna et al., 2022). The levels of SOD and CAT were expressed as the inhibition of NBT reduction per minute and H2O2 consumption per minute, respectively. The extent of lipid peroxidation and protein carbonylation were also estimated using the same protocols (Manna et al., 2022).

3.4.6. Analysis of pro-inflammatory cytokine levels

The level of IL-1β, IL-6, and TNF-α were measured in the blood serum and rat liver lysates using commercially available kits from RayBio®. The protocols outlined in the purchased enzyme-linked immunoassay (ELISA) kits were diligently followed, and all reagents, samples, and standards were prepared in accordance with the instructions provided in the kit's manual. In a nutshell, wells that had already been pre-coated with an antibody specifically targeting IL-1β, IL-6, and TNF-α were filled with 100 µL of samples and standards. After that, the mixture was let to sit at room temperature for 2.5 hours. 100 µL of biotin-conjugated antibody that is specific for IL-1β, IL-6, and TNF-α was then added to the well after the unbound compounds had been removed. The mixtures were then allowed to incubate for an additional hour at room temperature. Following the washing procedures, which entail draining each well of the liquid, adding 100 µl of Wash Buffer to the wells, and then draining the wells once again, we added 100 µl of prepared streptavidin solution to the wells and allowed them to incubate for 45 minutes at room temperature. Next, 100 µL of 3,3',5,5' tetramethylbenzidine (TMB) substrate solution was added. This was followed by another incubation at room temperature for 30 minutes. The amount of IL-1β, IL-6, and TNF-α bound in the first phase is directly correlated with the colour that develops as a result of this process. Each well received a little amount of the stop solution before the plate

was lightly tapped to ensure good mixing and the measurement of colour intensity at 450 nm. All standards and samples had their tests performed twice in order to reduce error.

3.4.7. Histopathological studies

A piece of liver tissue from experimental animals was thoroughly cleaned in ice-cold phosphate buffer with a pH of 7.4 in order to perform histological investigations. The tissue was then put in paraffin blocks for sectioning after being treated using a 10% formalin solution. The sections were suitably stained using hematoxylin and eosin (H&E) stains in line with the established protocol and imaged using Leica DFC 450C microscope (magnification 100x) (Dewanjee et al., 2013; Sahu et al., 2019).

3.4.8. Statistical Analysis

Experiments were performed in triplicate. The results of the experiment were represented by mean ± standard deviations (SD). With the aid of GraphPad InStat (version 3.05), GraphPad Software, USA, the findings were statistically analyzed using one-way ANOVA and Dunnett's t-test. P values under 0.05 were considered significant.

Chapter 4

Results and Discussions

Contents

4. Results and discussions

4.1. Effect on serum biochemical parameters

The toxic effect of $NaAsO_2$ causes damage in liver cells. Due to this, liver enzymes like ALT, ALP, AST releases into the blood serum. An increase in the level of these enzymes in sera indicates hepatocellular damage. The effects of *B. clausii* treatment on serum biochemical parameters of experimental rats were summarised in **Table 2**. $NaAsO_2$ (10 mg/kg) treated rats demonstrate significantly high serum levels of ALT, AST, ALP, LDH, CRP and CK in comparison to control group. Co administration of *B. clausii* (10^8 CFU) along with $NaAsO_2$ reduce this level to near normal state. However, it has been observed that treatment of experimental rats solely with the probiotic strain decreases the serum level of these enzymes more than the control group, which confirm it's beneficiary action on healthy liver.

Table 2. Effects on serum biochemical parameters in the experimental rats

Parameters	Group I	Group II	Group III	Group IV
ALT (IU/L)	23.80±3.23	76.35±0.6817	21.05±1.39	36.52±0.275
AST (IU/L)	75.215±3.23	168.47±0.6817	70.87±1.39	109.78±0.275
ALP (IU/L)	71.675±3.23	164.126±0.6817	68.53±1.39	106.36±0.275
LDH (IU/L)	172.56±3.23	311.77±0.6817	167.83±1.43	256.68±0.275
CRP (mg/L)	18.138±0.763	62.93±0.6817	16.485±1.14	53.49±0.294
CK (U/L)	95.4±3.23	793.91±0.6817	92.29±1.43	480.22±0.268

Values are expressed as mean ± SD (n=6). Values significantly ($p< 0.05$-0.01) differ from normal control. Group I: Normal control; Group II: Toxic control; Group III: *B. clausii*; Group IV: $NaAsO_2$ + *B. clausii*.

4.2. Effects on serum lipid profile

$NaAsO_2$ (10 mg/kg) administration in the experimental rats caused significant elevation of triglycerides and LDL level in blood serum and a slight increase in TC, when compared to control. As observed in the experiment *B. clausii* (10^8 CFU) co treatment with arsenic intoxication has a very little effect in normalizing TC and LDL parameters (**Figure 2**). It is also unable to reduce the elevated triglyceride level in the serum due to $NaAsO_2$ (10 mg/kg). The blood serum analysis reports reveals significant reduction of HDL in the toxic group of experimental rats. However, *B. clausii* (10^8 CFU) raises the HDL level to significant extent if administered alongside of $NaAsO_2$.

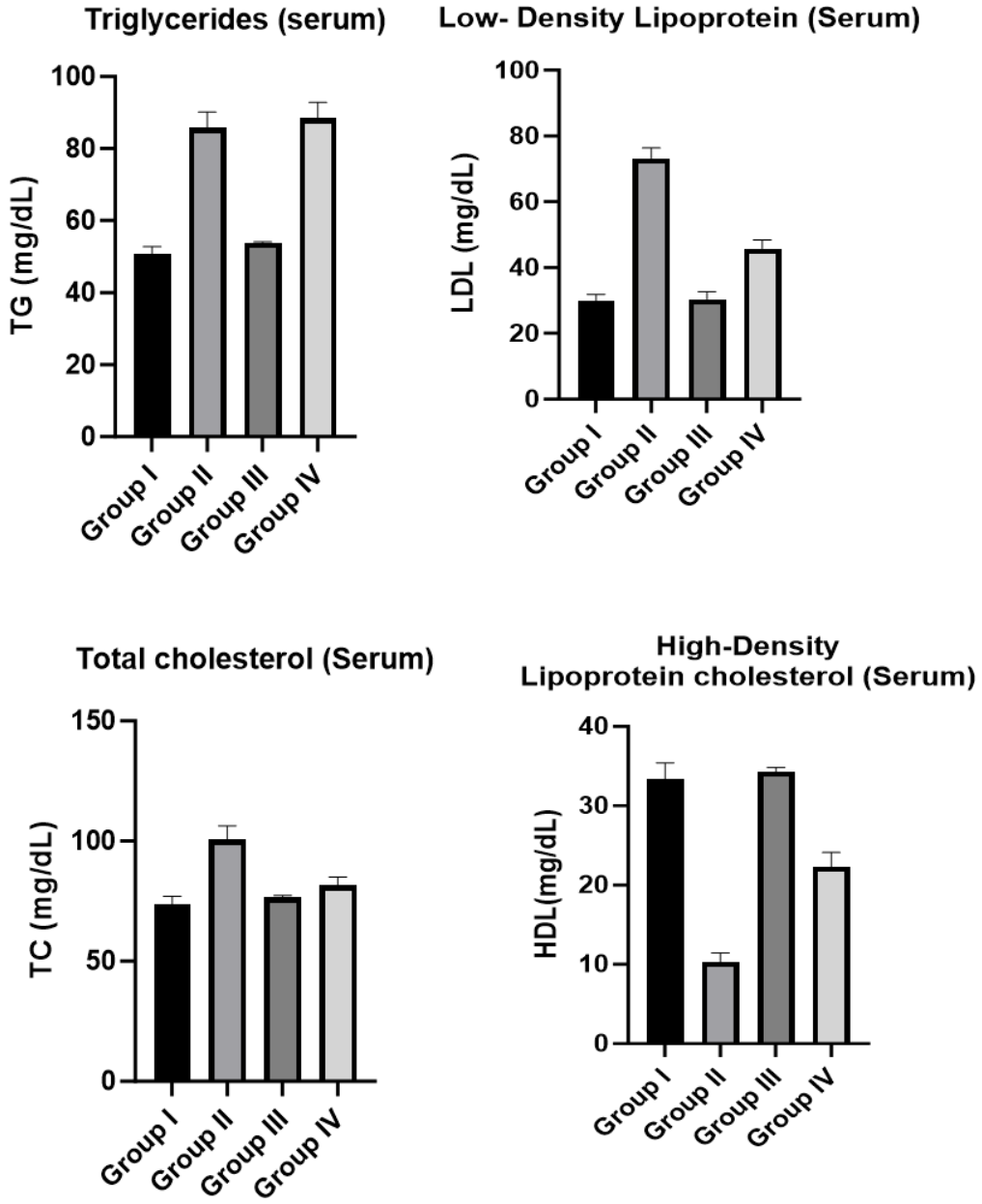

Figure 2. The effect on lipid profile in the serum of experimental rats of control group, $NaAsO_2$ treated group, only *B. clausii* treated group and $NaAsO_2$ + *B.clausii* treated group. Values are represented as mean ± SD (n =6). Values significantly ($p< 0.05$-0.01) differ from normal control. Group I: Normal control; Group II: Toxic control; Group III: *B. clausii*; Group IV: $NaAsO_2$ + *B. clausii*.

4.3. Effects on arsenic content in urine and hepatic cells

The effect of *B. clausii* treatment in arsenic content of the urine and hepatic tissue is summarised in **Table 3**. Significant amount of arsenic accumulation is observed in hepatic tissue in the toxic control rats treated with $NaAsO_2$ (10mg/kg). Though

co administration of probiotic decreases the arsenic content in hepatocytes, in comparison to control group it is still very high. Same observation was also found in urinary As content but in this scenario the rise in arsenic content in toxic group is much lower and treatment with *B. clausii* (10^8 CFU) almost restores the amount to near normal.

Table 3. Effects on arsenic content in urine and liver tissue of the experimental rats

Parameters	Group I	Group II	Group III	Group IV
Liver as (µg/g of tissue)	0.41±0.9	389.54±12.06	0.38±0.12	194.77±4.89
Urinary as (µg/g of creatinine)	0.245±0.14	3.6±0.64	0.22±0.09	1.053±0.21

Values are expressed as mean ± SD (n=6). Values significantly ($p< 0.05$-0.01) differ from normal control. Group I: Normal control; Group II: Toxic control; Group III: *B. clausii*; Group IV: $NaAsO_2$ + *B. clausii*.

4.4. Effects on biochemical and redox markers in liver

According to the results obtained in this study, $NaAsO_2$ (10 mg/kg) treated rats exhibited significant rise in ROS level of hepatic tissue (**Figure 3**). Though, *B. clausii* (10^8 CFU) attenuated this phenomenon but the effect was not in appreciable amount. In the liver of rats exposed to $NaAsO_2$ (10 mg/kg), significant upregulations in lipid peroxidation and protein carbonylation were found. The probiotic treated group significantly reduces this level to near normal by controlling the damage in liver tissue. By reducing the levels of antioxidant enzymes SOD, CAT and GSH; $NaAsO_2$ enhances oxidative stress, However, co-administration of *B. clausii* reverts back GSH and endogenous antioxidant level towards normal as found in control group rats.

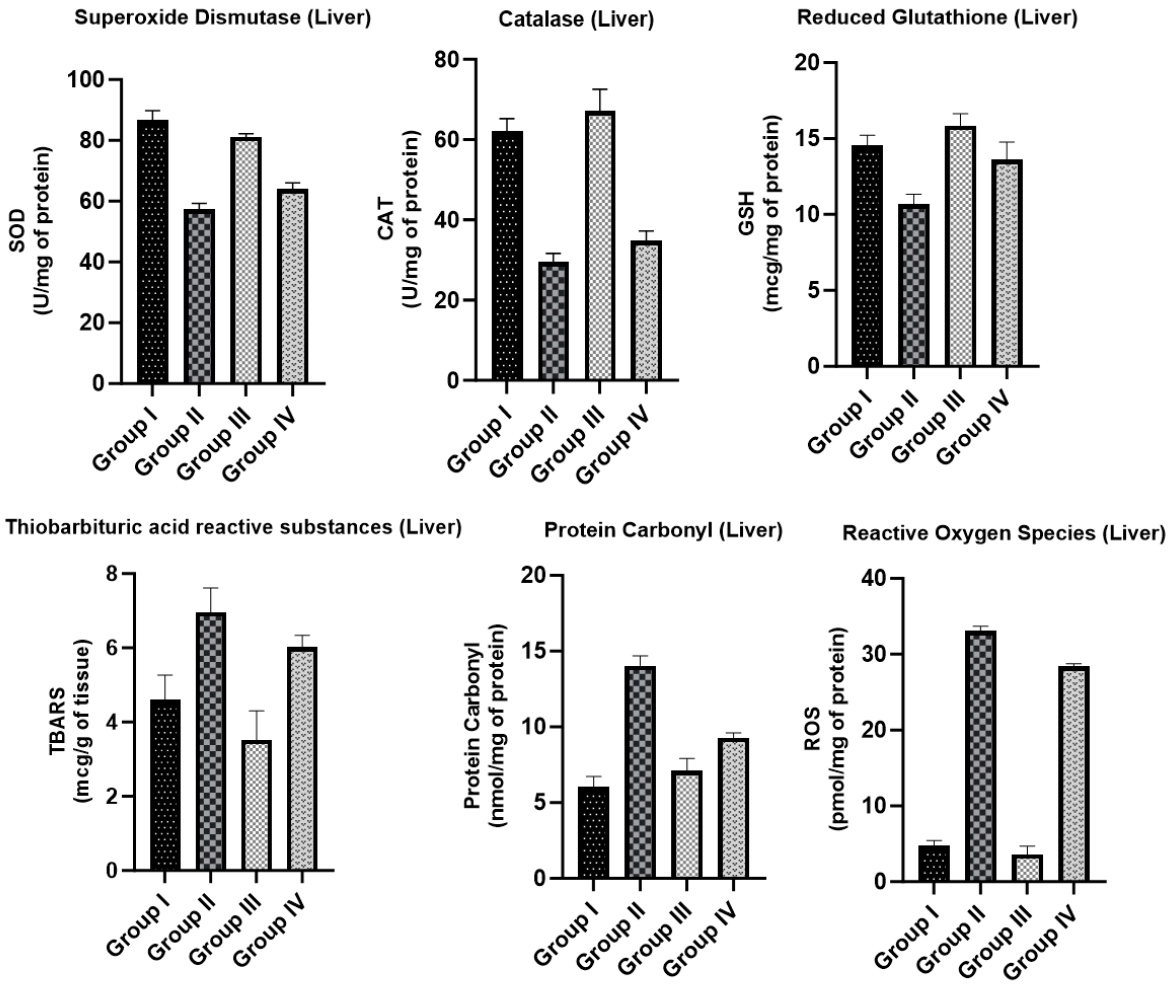

Figure 3. The effect on endogenous redox system, lipid peroxidation, protein carbonylation in control group, $NaAsO_2$ treated group, only *Bacillus clausii* treated group and $NaAsO_2$+*B.clausii* treated group *in vivo* in the liver of experimental rats. Values are represented as mean ± SD (n =6). Values significantly ($p< 0.05$-0.01) differ from normal control. SOD unit "U" is defined as inhibition (μ moles) of NBT reduction/ min. CAT unit "U" is defined as H2O2 consumption/min. Group I: Normal control; Group II: Toxic control; Group III: *B. clausii*; Group IV: $NaAsO_2$ + *B. clausii*.

4.5. Effects on pro-inflammatory cytokine levels

$NaAsO_2$ (10 mg/kg) intoxication triggers inflammation by enhancing pro-inflammatory cytokines in both blood serum and liver tissue as demonstrated in **Figure 4**. The levels of IL-1β, IL-6 and TNF-α cytokines were significantly increased in the rats of toxic control group in comparison to control group. This

phenomenon was controlled by treatment with *B. clausii* (10^8 CFU). The treatment group rats exhibits significant decrease in pro- inflammatory cytokines, though it was much higher than the control group. However, the experimental rats, receiving only *B. clausii* (10^8 CFU) were found to decrease the TNF-α levels more than the control group rats.

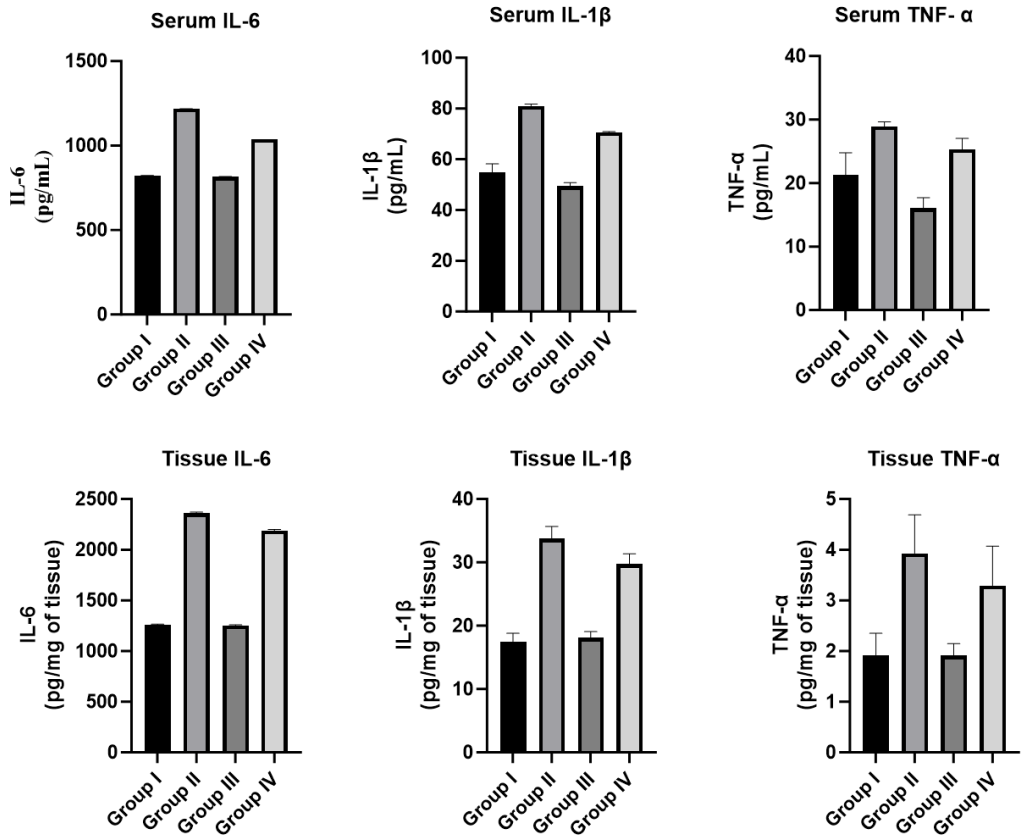

Figure 4. The effect on proinflammatory cytokines level in the serum of experimental rats of control group, $NaAsO_2$ treated group, only *B. clausii* treated group and $NaAsO_2$+*B.clausii* treated group. Values are represented as mean ± SD (n =6). Values significantly ($p< 0.05$-0.01) differ from normal control. Group I: Normal control; Group II: Toxic control; Group III: *B. clausii*; Group IV: $NaAsO_2$ + *B. clausii*.

4.6. Effects on the histopathology of the liver

Histological sections (100x) of the the experimental animals are given in **Figure 5**. It provides an overall idea of hepatic tissue damage caused by $NaAsO_2$ and its reverse effect by treatment with *B. clausii*. This potentiates the hepatocellular protective activity of the mention probiotic strain.

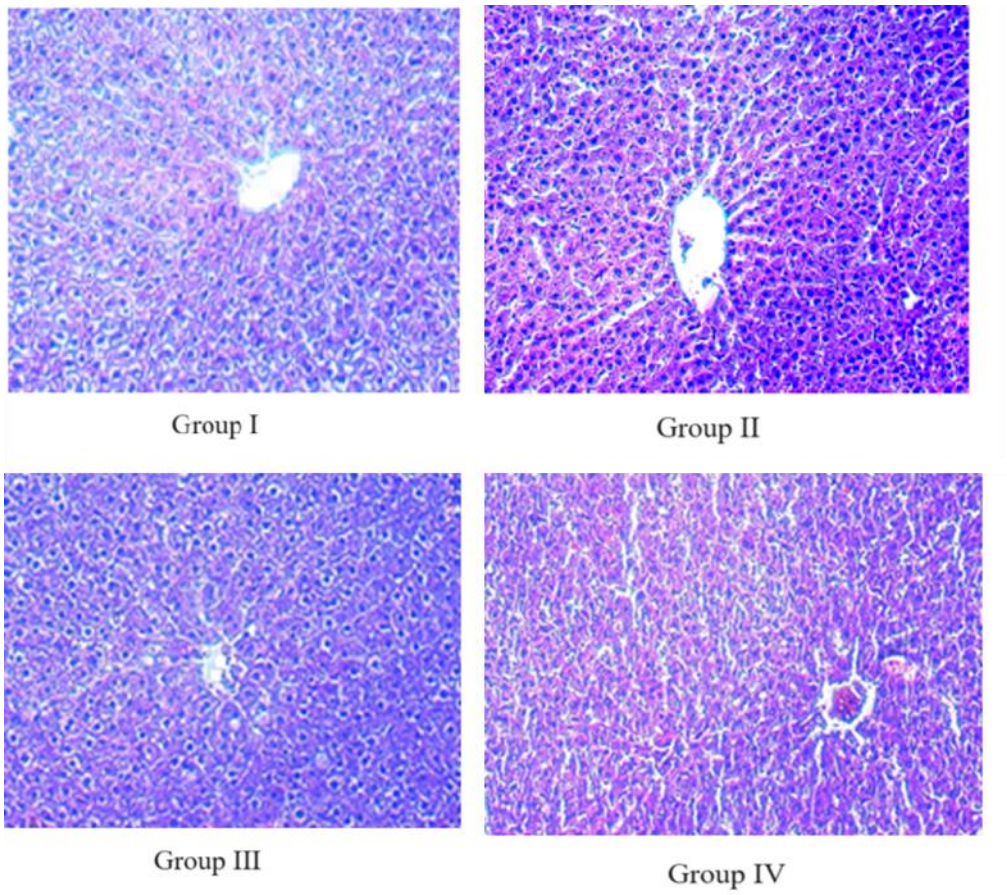

Figure 5. Histological sections of the livers of experimental rats of all the four groups. The liver sections of normal rats of Group I revealed normal portal vein and hepatocytes. $NaAsO_2$ (10mg/kg) treated rats of Group II exhibited dilated portal vein, vacuolated cytoplasm, cell apoptosis and leucocytes infiltration in the liver section. *B.clausii* (10^8 CFU) treatment reinstate $NaAsO_2$ mediated the aforementioned histopathological changes in the liver section of Group IV rats. Only *B.clausii* treated Group III exhibits similar condition of liver section as observed in control group.

Chapter 5

Conclusion

5. Conclusion

The present studies have been executed to evaluate the probable prophylactic role of *B.clausii* against As intoxication in the liver of experimental rat employing in vivo preclinical assays. Significant elevation of serum biochemical parameters (ALT, AST, ALP, LDH, CK) indicative for toxicological proceedings within the body, confirms the hepatoxicity caused by $NaAsO_2$. High level of these tissue specific enzymes in the blood serum confirms the hepatocellular damage in the toxic group. On the other hand observations of the experiments conclude decrease in level of these enzymes in serum with *B.clausii* treatment.

The lipid profile of the experimental rats were significantly disturbed by the $NaAsO_2$ administration. Toxic group shows huge elevation in LDL, TC and Triglycerides level and decrease in HDL level. The probiotic treatment does not show promising effect to normalise the lipid profile of the affected experimental rats who received As intoxication.

The arsenic accumulation in the liver tissue and urine significantly reduced after the treatment with *B. clausii*. $NaAsO_2$ primarily exhibits cytotoxic effect via ROS generation and provoking oxidative pathway. *B. clausii* exerts its cytoprotective action mainly through activation of antioxidant defence system and reducing the ROS. The experimental data of this experiment confirms this action. *B.clausii* significantly reduced the oxidative stress by increasing the levels of antioxidant enzymes (CAT, SOD) and GSH in As treated rats and normalizing them to near normal state. Significant downregulations in lipid peroxidation and protein carbonylation were also observed. Furthermore, it is responsible for reducing the ROS generation.

The pro-inflammatory cytokines were found to increase drastically due to As intoxication. The treatment with *B. clausii* reduced this level but not significantly.

Thus, it can be concluded that *B. clausii* exhibits hepatoprotective activity primarily via redox defence. Also, anti-inflammatory activity of this probiotic strain cannot be neglected. It has contribution in reversing As accumulation in liver. But, it has very little contribution in combating the rise in lipid profile due to arsenic mediated toxicity.

Chapter 6

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6. References

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