Effects of *Lactobacillus reuteri* and *Bacillus subtilis* against Arsenic-induced hepatotoxicity

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Submitted by

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CERTIFICATE OF APPROVAL

This is to certify that the thesis entitled "Effects of *Lactobacillus reuteri* and *Bacillus subtilis* against Arsenic-induced hepatotoxicity" has been carried out by **Ms. Shivangi Singh** 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 fulfilment 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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DEDICATED TO MY PARENTS

Mr. Ravindra Kumar Singh & Mrs. Rekha Singh

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PREFACE

Arsenic poses a significant risk to both humans and animals when present in food and drinking water. Being dependent on water for survival, aquatic creatures are especially susceptible to arsenic contamination. Additionally, animals that humans eat may collect arsenic, jeopardizing the safety of this food source. The main strategy for treating arsenic poisoning is to extract arsenic from bodily tissues. Chelation therapies have demonstrated some efficacy in treating acute arsenic poisoning; however, their usefulness in treating chronic instances remains questionable. Novel strategies are required because the majority of chelators have drawbacks and negative effects. Given the difficulties in using phytochemicals for treatment, investigating probiotics as the main therapy option would make more sense. Probiotics have been shown to benefit several liver conditions by lowering fibrosis, inflammation, and oxidative stress, by altering the usual gut flora's makeup and roles, which support the upkeep of a digestive tract in good condition. This enhances the synthesis of energy and guards the liver from damage. By triggering the body's antioxidant system, probiotics also lessen oxidative stress in the liver along with activating the synthesis of more antioxidant-producing molecules and enzymes. These also improve their capacity to function as antioxidants through a metal-chelating effect. To treat liver damage brought on by arsenic and produce more effective and secure treatment results, *Lactobacillus reuteri* and *Bacillus subtilis* are utilized. This thesis, which is divided into 6 individual chapters. A concise overview of the mechanism of arsenic-mediated hepatotoxicity and the treatment approaches are discussed in the **first chapter**. A thorough review of the literature on *Lactobacillus reuteri* and *Bacillus subtilis*, in their pertinent context, is presented in **Chapter 2**. The materials and techniques used to carry out the research are described in **Chapter 3**. The results are summarized in **Chapter 4**, which also includes pertinent commentary on the same. The study's conclusion is presented in **Chapter 5**, and a list of references supporting the research's significant findings is provided in **Chapter 6**.

CHAPTER 1

INTRODUCTION

Contents

1. INTRODUCTION

Arsenic (As), a trace metalloid, occurs naturally as a non-essential environmental toxin (Mirza et. al., 2014). It can be observed in the oxidation states as -3, 0, +3, and +5. Arsenious acids, arsenic acids, arsenites, arsenates, methyl arsenic acid (MAA), dimethylarsinic acid (DMAA), trimethyl arsine oxide (TMAO), and other forms constitute its environmental forms(Akter KF et. al., 2005). Arsenite (As III), a hard, deadly acid, and arsenate (As V) are the two most common forms of arsenic (Muzaffar et. al., 2023). While arsenite (As III) forms complexes with oxides and nitrogen chemical species, arsenate (As V) typically forms complexes with sulphides. In both reduced and oxidized states, Arsenic is mobilizable within the pH range of 6.5–8.5 (Tripathi et. al., 2007). Over 140 million people in at least 70 countries, including Afghanistan, Argentina, Bangladesh, Cambodia, Chile, China, India, Mexico, Mongolia, Myanmar, Nepal, Pakistan, Taiwan, Vietnam, sub-Saharan Africa, and the United States, are afflicted with Arsenicosis (caused by prolonged exposure of Arsenic), which is primarily caused by trivalent inorganic arsenicals (arsenite) in subterranean water (Brinkel et. al., 2009; Das et. al., 2010). Arsenites are absorbed through the gastrointestinal tract and disseminated throughout the body after being consumed orally as tainted drinking water (Ratnaike, 2003). Arsenicals could, however, also enter the body through skin cells and through the act of inhalation and exhalation. Arsenic takes part in cellular redox mechanisms that result in the creation of excess reactive oxygen species (ROS) post-bioaccumulation. Furthermore, Arsenic has an augmented affinity towards thiol groups, which corresponds to an additional mechanism for the development of oxidative stress in patients suffering from arsenicosis (Rizwan et. al., 2014). Apoptosis is triggered by the escalating oxidative stress and the depletion of the endogenous antioxidant response during arsenic intoxication. Besides damaging cellular macromolecules, oxidative stress additionally ends up in peroxidative destruction of membrane lipids, oxidative harm to DNA, and carbonylation of proteins, all of which influence cellular pathology (Dutta et. al., 2014). Arsenic impacts nearly every vital organ and tissue, including the bloodstream, the liver, kidneys, lungs, heart, testes, and brain. Even though Arsenic-induced toxicity is a ubiquitous issue, there isn't any trustworthy, precise, and safe treatment of the same (Dua et. al., 2015). The primary treatment approach to alleviating Arsenic intoxication is believed to be Chelation Therapy; however, numerous adverse effects of chelators, which include the elimination of vital metals and the redistribution of Arsenic, restrict their clinical efficacy (Dua et. al., 2016). Potential therapeutic value in terms of lowered morbidity and mortality for chronic Arsenic intoxication is mainly undetermined despite the fact that

chelation for arsenic chronic intoxication may expedite metal excretion and diminish metal concentration in specific tissues (Kosnett, 2013). Utilizing the prophylactic capabilities of natural antioxidants to combat Arsenic intoxication makes good sense, given the crucial role that oxidative stress plays in the genesis of Arsenic toxicity (Dua et. al., 2016). Probiotics have been shown to possess potential antioxidant properties and can attenuate liver damage which makes them a good option for the treatment (Dewanjee et. al., 2022).

Probiotics consist of living, non-pathogenic microorganisms, such as several commensal bacteria, that, when administered in appropriate amounts, confer benefits to the host's health and prevent disease (Dewanjee et. al., 2022). Probiotic strains that are precisely described, maintain their viability in a formulation thus not altering its shelf life, and have at least one successful clinical study to support their efficacy and safety are considered prominent traits of those strains (Binda et. al., 2020). Lactic acid bacteria, which comprises various kinds of strains of Lactobacillus, Bifidobacterium, Streptococcus, and Enterococcus, make up a major chunk of probiotics (Linares et. al., 2017). Among them, Lactobacillus and Bifidobacterium are commonly found in various fermented milk goods and are also advertised and promoted commercially as nutritious foods or nutraceuticals (Taye et. al., 2021).

Probiotics have gained attention as possible nutraceuticals with claims to enhance health and fend off disease (Cunningham et. al., 2021). Several studies aimed at the gut found that the permeability of the gut barrier may be heightened by gut dysbiosis, leading to the translocation of bacteria and the leakage of gut-derived products (Meng et. al., 2018). These substances penetrate the liver through the portal vein and potentially cause elevated levels of oxidative stress and inflammation, endangering the hepatic health of the individual (Yip et. al., 2018). Pathogenesis of liver diseases can be influenced by changes in the gut microbiota and associated metabolites, and vice versa (Qin et. al., 2014). It has been demonstrated that probiotic strains such as Lactobacillus alleviate liver complications by modifying the major bile acids, enhancing intestinal permeability, and transforming gut flora (Meng et. al., 2018). Probiotics can also assist the liver in recovering from illnesses, and oxidative stress-induced acute liver toxicity (Dewanjee et. al., 2022). and xenobiotic-induced hepatotoxicity while reestablishing healthy liver function (Eslamparast et. al., 2013). Taking into consideration all the aforementioned findings, this research aims to study and explore the hepatoprotective roles of probiotic *Lactobacillus reuteri (L. reuteri)* and *Bacillus subtilis (B. subtilis)* and how these probiotics alleviate the detrimental effects of xenobiotic toxicity, in this case, Arsenic, on rats.

1.1 **Environmental fate and arsenic infiltration**

As is ubiquitous to the environment, however, the quantities vary, which are impacted by the proximity of places with anthropogenic or natural spoilage (Flora, 2011). Geological formations, which include the deposition of arsenic in sedimentary rocks, volcanic rocks, and soils, constitute one of the most significant natural sources of arsenic. (Beck et. al., 2017). Anthropogenic activities such as mining, coal, and petrochemical extraction have been linked to arsenic accumulation in groundwater in 54 nations globally (Punshon et. al., 2017).

For a large number of individuals, consuming food and water are the principal oral avenues by which arsenic enters the body (Clemente et. al., 2016). A recent study employing over 50,000 consolidated data points revealed that between 94 million and 220 million people worldwide are potentially exposed to unacceptably elevated levels of groundwater arsenic (Podgorski and Berg, 2020). A maximum of 10 µg/L of Arsenic in drinking water is considered the threshold but anything more than this quantity, is alarming (WHO, 2019). The acute toxicity of soluble inorganic arsenic is extremely dangerous. Prolonged exposure to arsenic (Arsenicosis) may happen from long-term inorganic arsenic ingestion (Ramly et. al., 2023). The Indian state of West Bengal was the first to discover the problem of Arsenicosis caused by contaminated groundwater in 1984 (Garai et. al., 1984; Saha, 1984). A survey report from 2001 indicates that approximately 150 million people in the combined areas of West Bengal and Bangladesh are exposed to risk from arsenic-contaminated groundwater (Rahman et. al., 2001). It's interesting to note that the rollercoaster of effects and side effects continues to remain functional today (Waxman et. al., 2001).

Depending on the duration of exposure, effects can take years to manifest. These involve skin lesions, peripheral neuropathy, gastrointestinal problems, diabetes, cardiovascular disease, developmental damage, and carcinoma of the skin (Khargas et. al., 2015). Other clinical features that correspond to arsenic toxicity include peripheral vascular disease (Blackfoot disease), noncirrhotic portal hypertension, hepatomegaly, respiratory and renal involvement, poor obstetrical outcome, and haematological disturbances (Maharjan et. al., 2005). The trivalent form of arsenic, referred to as arsenite, is primarily accountable for the consequences. It binds with sulfhydryl groups that inhabit a variety of necessary molecules, causing inactivation and disruption of bodily processes. Arsenic is generally ingested in its pentavalent form, or Arsenate, even though its toxicities are mostly associated with its trivalent state (Sengupta et. al., 2008). According to recent studies, the following is the order in which acute arsenic intoxication takes place: Mono Methylarsonic Acid(III)>Trivalent Arsenic(III)>Pentavalent Arsenic(V)> Dimethylarsinic Acid (V)> Mono Methylarsonic Acid

(V), with Mono Methylarsonic Acid also known as MMA(III) being the most hazardous metabolite (Kile et. al., 2011).

1.2 Underlying mechanism of arsenic toxicity

Arsenic has been associated with modifications in several cellular functions, such as the impairment of DNA repair, decreased immunosurveillance, growth factor expression shifts, apoptosis resistance, restriction of cell cycle screening proteins, transformed DNA methylation, and elevated levels of oxidative stress (Lantz and Hays, 2006). Oxidative stress is one of the most researched mechanisms of action (Calatayud et. al., 2013). By cycling between the oxidation states of metals such as iron, interfering with intracellular antioxidant levels, or triggering inflammation, arsenic may bring about oxidative stress. This may culminate in the continuous existence of cells that create radicals and/or growth factors (Flora, 2011). According to *in vitro* studies, As(III) alters intercellular junctions, increases permeability through the cell monolayer, and causes oxidative stress and pro-inflammatory response in body cells(de Matsuoka et. al., 2020). The harmful effects of arsenic are significantly influenced by its metabolism. Several mammals, if not all, methylate inorganic arsenic in numerous instances (Hughes, 2002). Arsenic's oxidation state and chemical form contribute to its acute toxicity. A two-electron reduction of pentavalent arsenic to trivalent arsenic and oxidative methylation to pentavalent organic arsenic are the two phases in the process of metabolism of inorganic arsenic (Thomas et. al., 2001). The mechanism for toxicity is different for both pentavalent and trivalent arsenic (Hughes , 2002). In the case of pentavalent arsenic toxicity, arsenate replaces phosphate, which is a key component in a majority of biochemical reactions like glycolysis, gluconeogenesis, etc (Tawfik and Viola, 2011). This happens because both arsenate and phosphate share similar structural composition and properties (Nemeti et. al., 2012). For instance, *in vitro* reactions among arsenate and glucose and gluconate result in the formation of glucose-6-arsenate and 6-arsenogluconate, respectively (Gregus, 2009). These substances are similar to glucose-6-phosphate and 6-phosphogluconate, in that order. Both glucose-6-phosphate and glucose-6-arsenate can block hexokinase and serve as substrates for glucose-6-phosphate dehydrogenase (Nemeti et. al., 2010). In the sodium pump and anion exchange transport mechanism of the human erythrocytes, arsenate is also capable of taking the place of phosphate (Kenney and Kaplan, 1988). Arsenolysis is a process through which arsenate uncouples the *in vitro* generation of adenosine-5′-triphosphate (ATP) (Thomas, 2010). During glycolysis, arsenolysis may take effect at the substrate level. The enzymatic linkage between phosphate and d-glyceraldehyde-3-phosphate yields 1,3-biphospho-d-glycerate, which is one step in the glycolytic pathway (Nemeti et. al., 2002). In

this reaction, arsenate can take precedence over phosphate to generate 1-arsenato-3-phospho-d-glycerate, an anhydride. When phosphate is abundant during glycolysis, only then is ATP generated, but arsenate's presence does not guarantee the same (Drobna et. al., 2009). Arsenolysis may happen at the mitochondrial level when oxidative phosphorylation occurs (Nemeti et. al., 2002). Compared to ATP, which is produced during oxidative phosphorylation, ADP-arsenate hydrolyses more readily (Hughes, 2002). Arsenolysis reduces the amount of ATP produced *in vitro* at both the substrate and mitochondrial levels by substituting arsenate for phosphate in the enzymatic processes (Tawfik and Viola, 2011). In cellular systems, arsenate has been reported to diminish ATP levels. After repeated exposure to arsenate *in vitro*, human and rabbit erythrocytes have shown alleviated ATP levels. Arsenite, however, fails to diminish ATP levels in human erythrocytes (Hughes, 2002). The toxicity and the mechanism of action discussed above was of pentavalent arsenic which is the most abundant form of arsenic in the environment. However, pentavalent Arsenic, when transforms itself, i.e., undergoes biotransformation, gets reduced and converts into an even more toxic form i.e., the trivalent arsenite (Thomas et. al., 2001; Hughes et. al., 2011). Because of their strong affinity toward SH groups, the trivalent arsenites As (III) and the ensuing trivalent methylated arsenic metabolites are extremely noxious and interfere with the operation of essential biological proteins (Kitchin and Wallace, 2008). The most prevalent thiol molecule in the body, Glutathione (GSH), contributes to pentavalent Arsenic reduction *in vivo*(Csanaky, Gregus, 2005). Glutamyl-cysteine synthetase and glutathione synthetase (GS), two ATP-dependent enzymes, serve as catalysts for the generation of this tripeptide (Jan et. al., 2015). The primary site of arsenic detoxification is the liver because of its high glutathione content(Susan et. al., 2019). However, the liver is particularly vulnerable to the detrimental effects of arsenic poisoning at elevated concentrations (Ratnaike, 2003). Trivalent arsenic that has been methylated has been demonstrated to cause hepatotoxicity by employing interactions with hepatocyte DNA and protein receptors. This results in diminishing methylation efficacy of the liver, which causes arsenic to accumulate in the body (Sharma et. al., 2014). One of the principal indicators of arsenic poisoning is liver enlargement, which is followed by recurrent dyspepsia and ultimately hepatic fibrosis (Guha, Mazumder, and Dasgupta, 2011). Chronic exposure to arsenic induces hepatoportal sclerosis, which frequently coexists with local vascular impairment (Banerjee et. al., 2017). Cirrhosis, hepatocellular infiltration, degenerative lesions, centralized necrosis, ascites, and cancer of the liver are additional ailments attributed to prolonged arsenic exposure. Sustained exposition to arsenic has been demonstrated to lead to malignant cell types proliferating in the liver of rats and cause hepatic neoplastic ulcers in

female Swiss mice (Lu et. al., 2001). It has been speculated that methylated arsenicals promote DNA damage, and that arsenic's liaisons to zinc finger proteins, which play a crucial role in DNA repair, degrade DNA repair pathways and trigger carcinoma of the liver (Muenyi et. al., 2015). Following arsenic exposure, the blood has been found to contain liver enzymes such as aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) as a result of cellular enzyme leakage and cellular integrity loss (Susan et. al., 2019). Due to an increase in reactive oxygen species production, arsenic intoxication accelerates lipid peroxidation in the liver (Singh et. al., 2011). It has been discovered that exposure to arsenic elevates the levels of lipid peroxidation markers such as malondialdehyde and thiobarbituric acid (TBAR) (Medda N et. al., 2021). Glutathione peroxidase (GPx) activity and glutathione (GSH) levels are both alleviated in arsenic poisoning, despite the fact that both enzymes are essential for regulating oxidative stress (Hall et. al., 2013). Arsenic-mediated inhibition of glutathione reductase, arsenic-induced inhibition of glutathione production, or arsenic-GSH complexation may collectively contribute to the depletion of glutathione levels in the liver (Laborde, 2010). A metalloenzyme which is a component of the cell's antioxidant mechanism is superoxide dismutase (SOD) (Singh et. al., 2017). Because of the excess of free radicals, arsenic poisoning impairs SOD activity within the tissues of the liver and kidneys (Susan et. al., 2019). Another enzyme called GPx functions as an antioxidant by neutralizing hydrogen peroxides and lipid peroxides. According to recent research, arsenic promotes superoxide radicals to flourish, which in turn encourages a rise of hydroxyl radicals, resulting in greater ROS accumulation and genotoxicity (Jomova et. al., 2011).

1.3 Available treatment interventions against arsenic-induced toxicity

1.3.1 Chelation therapy

As we know, As is a metalloid, scientists have well-explored the possibilities of employing ligands to form a complex with Arsenic in order to remove it from the body’s systems. They have termed this approach as the ‘Chelation Therapy’ and the molecules aiding in this procedure are referred to as Chelating Agents (Susan et. al., 2019). The chelates thus formed, prevent the interaction between Arsenic and its biological targets like proteins and nuclear material and facilitate its excretion from the system (Flora et. al., 2007). The primary hurdle in eliminating arsenic is deciding on a suitable chelating agent. An ideal chelating agent should be lipid-soluble, non-toxic, have a strong affinity towards arsenic, and should be excreted from the body in its complex configuration (Aaseth et. al., 2014). It is crucial to consistently remove the complex generated between arsenic and the chelating ligand from the system as the complexation reactions tend to reach equilibrium (Clarke et. al., 2012). For the chelating ligand

to integrate into the cellular compartment and chelate arsenic, lipid solubility is crucial (Sears, 2013). However, previous research investigations have demonstrated that water-soluble chelating compounds are less perilous than their lipophilic analogues(Smith SW, 2013). British anti-Lewisite (BAL), also known as dimercaprol or 2,3-dimercapto-1-propanol, was the first chelating agent to be used for the removal of arsenic(Kosnett MJ, 2013). A few chelators used for the treatment of Arsenic Toxicity are listed below in **Table 1**.

Table 1. Effects of Synthetic Chelating Agents against Arsenic-mediated toxicity

Chelating agents	Salient features	References
British anti-Lewisite (BAL)	BAL has thiol groups which form a complex with Arsenic which aids in eliminating the resultant chelate via urine.	Kosnett, 2013; Susan et. al., 2019.
2,3-dimercaptopropane-1-sulphonic acid (DMPS)	Water soluble BAL derivative; forms an insoluble Arsenic complex by employing an organic transport pathway to get into the cell.	Aaseth et. al., 2014; Yadav and Flora, 2016.
Meso-2,3-dimercaptosuccinic acid (DMSA)	Uses 2 thiol groups to chelate Arsenic. However, it has the limitation of not being able to seep into the cell membrane, and thus, it cannot chelate Arsenic in the intracellular compartments.	Flora et. al., 2007; Kosnett, 2013; Susan et. al., 2019.
D-Penicillamine	Sulfhydryl groups form a chelate with Arsenic and eliminate it via urine and faeces.	Flora et. al., 2007; Aaseth et. al., 2014.
Mono isoamyl DMSA (MiDMSA)	Lipophilic derivative of DMSA has a high affinity towards Arsenic and chelates both extracellular and	Pachauri et. al., 2013, Aaseth et. al., 2014.

	intracellular arsenic through the sulfhydryl groups.	
DMSA combined with Mono cyclohexyl DMSA (MchDMSA)	Alleviates ROS and elevates the levels of anti-apoptotic proteins as well as restores mitochondrial protection.	Flora et. al., 2007; Pachauri et. al., 2013; Aaseth et. al., 2014.
Zinc and N-acetyl cysteine	When used combinedly, provides enhanced protection against D-aminolevulinic acid dehydratase (ALAD) inhibition by arsenic and alleviates oxidative stress.	Modi et. al., 2006; Susan et. al., 2019.

As per the existing literature available on the use of chelating agents, it is evident that a vast majority of chelators have different downsides and adverse effects. Therefore, it is certainly not feasible to successfully manage arsenic-induced toxicity with these chelating substances.

1.3.2. Anti-oxidant therapy utilizing flavonoids and other phytochemicals

As oxidative stress is the primary cause of the majority of arsenic's detrimental effects, antioxidant therapy has emerged as a successful means to mitigate arsenic's harmful consequences. Since arsenic disrupts the intracellular antioxidant framework, pro-oxidant stress triggered by arsenic can be countered by exogenous antioxidant supplementation. Numerous flavonoids have been demonstrated to have antioxidant abilities, which could potentially mitigate the harmful effects of arsenic (Susan et. al., 2019). Along with flavonoids, several plant extracts have been studied extensively to investigate their ability to mitigate the toxicity associated with heavy metal poisoning because they are rich in chelating and anti-oxidant properties (Boonpeng et. al., 2014). Numerous plant species have been examined to figure out how effective they are at eliminating arsenic (Gupta et. al., 2015). **Table 2** comprises a list of various flavonoids and phytochemicals used to mitigate Arsenic-induced impairments and toxicities.

Table 2. Therapeutic intervention of flavonoids and phytochemicals used against Arsenic induced toxicity

Anti-Oxidant agents	**Salient features**	**References**

Naringenin	Restores antioxidant enzymes and liver serum biomarkers; minimizes DNA breakage and undoes pathological alterations brought forth by arsenic poisoning in the kidney and liver tissues.	Mershiba et. al., 2013
Silymarin	Lowers the synthesis of conjugated dienes and reduces lipid peroxidation.	Bongiovanni et. al., 2007
Silymarin and Naringenin	Restores inflammatory markers like SOD, Catalase, and GSH; lowers malonaldehyde and arsenic concentration in hepatic tissues.	Jain et. al., 2011
Epigallocatechin-3-gallate (EGCG)	Suppresses LDH, CK, CK-MB, and AST levels; inhibits ROS production; modulates calcium homeostasis; reverses cardiac toxicity; mitigates immune suppression, inflammation, and apoptosis triggered by sodium arsenite; and lowers oxidative stress by activating Nrf2 signaling.	Biswas et. al., 2017; Mershiba et. al., 2013; Guvvala et. al., 2017; Han et. al., 2017.
Allicin, allyl cysteine, alliin, allyl disulfide	Scavenge superoxide ions and hydroxy radicals.	Chung, 2006
Quercetin	Reduces ROS production and proliferation and prevents oxidative stress.	Mishram and Flora], 2008
Proanthocyanidin	Alleviates the toxicity in the reproductive organs, stimulates the expression of transcription factors such Nrf2, HO1, GST, and NQO1 that are linked to detoxification processes.	Li et. al., 2015

1.4. Objectives of the study

As everyone knows, Arsenic is a major cause of illnesses and systemic toxicities in an enormous percentage of people worldwide. Antioxidant and Chelation Therapy are two effective treatment approaches, but they have drawbacks as well, such as removing important metal ions in addition to arsenic. Although herbal extracts have been proven to affect hepatotoxicity, they have also demonstrated several unfavourable side effects. Given the present predicament, a novel approach to treating arsenic-induced hepatotoxicity is required. Probiotics have demonstrated enormous promise in modulating the gut microbiome and serving as antioxidants and immunomodulators. However, further research is essential to fully discover these perks, and in this study, two distinct probiotic strains—*Lactobacillus reuteri* and *Bacillus subtilis*—are examined to determine their safeguarding against hepatotoxicity.

The objectives of this research study are enlisted below:

- To study the hepatoprotective effects of *Bacillus subtilis* & *Lactobacillus reuteri* against experimentally induced hepatotoxicity using Arsenic.
- To study the anti-inflammatory properties of *Bacillus subtilis* & *Lactobacillus reuteri.*
- To evaluate the toxicity-ameliorating benefits of probiotic therapy by using strains *Lactobacillus reuteri* and *Bacillus subtilis* and study its prospects concerning xenobiotic toxicity.

CHAPTER 2

LITERATURE REVIEW

Contents

2. LITERATURE REVIEW

Probiotics are living microorganisms that offer the host substantial health advantages when administered in sufficient amounts. Although the notion of utilizing probiotics for medical purposes is not novel, interest in the practice has grown substantially in recent years (Islam, 2016). This could be partly attributed to the rise of antibiotic resistance, especially when treating gastrointestinal (GI) disorders, and a growing consumer appetite for natural health boosters (Kechagia et. al., 2013). Probiotics are live, non-pathogenic microorganisms, such as certain commensal bacteria, that, when given in the right dosage, provide benefits to the host's health and prevent disease (Dewanjee et. al., 2022). Probiotic strains that are well-defined, maintain their viability in a formulation for its shelf life, and have at least one successful human study to support their efficacy and safety are considered prominent traits (Binda et. al., 2020). Liver health is significantly maintained by microbes in the gut (Meng et. al., 2018). Therefore, a potential strategy for hepatoprotection is to modify the gut microbiota. Probiotics protect the liver from damage by replenishing the gut microbial community, which strengthens the intestinal wall, decreasing the spread of bacteria and epithelial encroachment and reducing endotoxemia, according to an expanding body of research (Eslamparast et. al., 2013) (Twardowska et. al., 2022). They have the ability to both boost the immune system and initiate the synthesis of antimicrobial peptides at the same time (Eslamparast et. al., 2013) (Sánchez et. al., 2013). Probiotics can also lessen inflammatory and oxidative liver damage (Eslamparast et. al., 2013) (Meng et. al., 2018).

One of the numerous diverse and nonsporulating facultative anaerobic bacteria in the genus Lactobacillus which is assimilation-prone is *Lactobacillus reuteri*. This genus participates in food fermentation and is present in varying levels in the gastrointestinal tract of humans and animals, depending on the host's age, species, and gut location (Duar et. al., 2017). At the same time, the *Bacillus* species (sp) exhibit impressive resistance to physical and chemical stimuli, such as radiation, heat, and toxic chemicals, which are typically thought to be detrimental to microbes (Fan et. al., 2019).

After studying several strains carefully and comprehensively, these two were chosen out of the numerous strains present in the market to carry out further research, in order to determine their clinical significance and medical applications of these promising strains, on several kinds of health conditions. In this study, it has been aimed to determine the positive effects of these strains on human health.

2.1 Experimentally explored physiological benefits of *Lactobacillus reuteri*

2.1.1 Role of *Lactobacillus reuteri* in Hepatoprotection

The gut's byproducts, including microbiological components, continuously remain in contact with the liver (Balmer et. al., 2014) (Nakamoto, 2014). Pattern recognition receptors, which recognize bacterial components like LPS and trigger inflammatory pathways, are expressed by hepatocytes (Takeuchi, Akira, 2010). A growing body of investigation has shown that gut bacteria and hepatic receptors, such as Toll-like receptors (TLR), are more likely to interact when there is a poor gut barrier, or "leaky gut"(Paolella et. al., 2014). Furthermore, TLR signals are essential for controlling the liver's innate immune response (Nakamoto, Kanai, 2014). For example, LPS activates Kupffer cells' TLR4, which results in the production of pro-inflammatory cytokines like TNF-α and hepatocyte damage. Therefore, the imbalance in the gut-liver axis triggers an inflammatory response that results in the development and advancement of liver problems (Cui et. al., 2019), including hepatic encephalopathy (Dalal et. al., 2017), non-alcoholic fatty liver diseases (Boursier et. al., 2016), and liver cirrhosis (Shi et. al., 2017). Conversely, it has been demonstrated that probiotic-induced alterations in the gut microbiota may benefit liver health (Lo et. al., 2014). Hence, a microbial intervention-based treatment is a promising way to enhance the liver's disease process (Chen et. al., 2014). Probiotics, like Lactobacillus, can enhance the host's immune system and metabolic processes while inhibiting the growth of ailments. Numerous strains of *Lactobacillus reuteri* are found in the mammalian gut and are beneficial to health (Mu et. al., 2018). Different studies have shown that pretreatment with *L. reuteri* DSM 17938 decreased tissue abnormalities in the last segment of the ileum and liver, as well as gamma-glutamyl transferase, blood levels of alanine aminotransferase and aspartate aminotransferase (Jiang et. al., 2021). Furthermore, the abundance of several potentially pathogenic taxa, including Actinomycetales, Coriobacteriaceae, Staphylococcaceae, and Enterococcaceae, was reduced by *L. reuteri* DSM 17938. Moreover, it reduced the hepatic production of various inflammatory genes (Wong et. al., 2017). Furthermore, *L. reuteri* DSM 17938 alleviates liver failure via a number of mechanisms, such as the enhancement of viral protein association with cytokines and cytokine receptors, the inhibition of retinol metabolism and the peroxisome proliferator-activated receptor (PPAR) signaling pathway, and the central carbon metabolism in the cancer signaling pathways (Hsieh et. al., 2013) (Hsu et. al., 2017).

2.1.2 Role of *Lactobacillus reuteri* in Obesity Control

Obesity is defined as a detrimental build-up of excess fat in the body that leads to a reduction in health and quality of life. It has been established that there is a direct link between obesity

and the Western diet (Martinez et. al., 2017). Obesity is a major health worldwide because it can cause the onset of hypertension, diabetes mellitus, insomnia, and coronary artery disease. Additionally, there is now a significant association between the microbiota of the gut and obesity (Daniali et. al., 2020). Specifically, changes in the abundance of gut microbiota play a role in obesity. Probiotics have therapeutic value in treating dysbiosis of the gastrointestinal tract, which is typified by an abnormal microbial composition, reduced intestinal barrier permeability, and activated inflammation (Bäckhed et. al., 2012). Notably, studies have looked at Lactobacillus to see if it can balance the microbiota in the intestines and manage obesity (Tchernof, Després, 2013) (Dabke et. al., 2019). The *L. reuteri* MG5149 strain was shown in an investigation to lower adipocyte size in epididymal tissue, weight of the body, and fat tissue weight. Furthermore, it decreased the expression of lipogenic protein molecules in certain tissues, including fatty acid synthase, adipocyte-protein 2, peroxisome proliferator-activated receptor, CCAAT/enhancer-binding protein, and AMP-activated protein kinase. Furthermore, it decreased the accumulation of fat by elevating the phosphorylation of AMP-activated protein kinase and acetyl-CoA carboxylase (Choi et. al., 2021). Additionally, the administration of *L. reuteri* JBD30l reduced the amount of free fatty acids (FFAs) in the gut fluid of the small intestine, hence decreasing intestinal FFA absorption and elevating faecal FFA excretion (Chung et. al., 2016). Contradictory findings, however, suggest that *L. reuteri* might strain-dependently promote the advancement of obesity. The association was verified when it was found that the presence of vancomycin-resistant *L. reuteri* in the gut microbiota predicted an elevation in body weight after vancomycin treatment (Million et. al., 2013). Due to the amount of fructose consumed, several studies have linked *L. reuteri* abundance to weight gain (Huerta-Ávila et. al., 2019). Fructose molecules may be used by *L. reuteri* as an avenue of energy to accelerate its development, which would lead to an elevated uptake of fructose and a boom in the generation of intermediary molecules, which would result in the production of triglycerides (Huerta-Ávila et. al., 2019). Probiotic-mediated weight reduction has been linked to several possible mechanisms of action, including altered gut microbiota layout, production of short-chain fatty acids (SCFA), disruption of the metabolism of bile acids in the host, and regulation of energy homeostasis and satiety. In actuality, one of the probiotic bacteria that can carry out these methods to regulate obesity is *L. reuteri* **(Figure 2)**. Conversely, food components that promote the growth of *Lactobacillus reuteri* in the stomach lead to a reduction in body weight (Cerdó et. al., 2019). To find out if weight loss is only due to an increase in *L. reuteri* or if it is substrate-dependent, additional investigation is required.

2.1.3 Role of *Lactobacillus reuteri* in Insulin Sensitivity and Glucose Homeostasis

Hyperglycaemia and insulin resistance linked to a decreased incretin response, subclinical inflammatory reactions, and decreased glucose tolerance are the hallmarks of type 2 diabetes mellitus (T2DM) (Cerdó et. al., 2019). In experimental animals, the gut microbiota can be modulated to control resistance to insulin (Cani et. al., 2007). Moreover, a reduction in systemic and hepatic inflammation as well as portal lipopolysaccharide, or LPS, endotoxin concentrations is associated with improved intestinal barrier function (Cani et. al., 2007). Regular administration of *L. reuteri* SD5865 boosted glucose-stimulated GLP-1 and GLP-2, enhanced insulin sensitivity, and enhanced insulin secretion by elevating incretin release, as shown by Simon et. al., 2015.

2.1.4 Role of *Lactobacillus reuteri* in Immunomodulation of the Gut Microbiota

A major contributing reason to the rise in the prevalence of several GI illnesses has been shown to be alterations in the intestinal flora (Isolauri, 2001) (Tong et. al., 2007) (McFarland, Dublin, 2008). The effectiveness of probiotics varies depending on the strain, and each strain may support host health differently. Probiotic bacteria have three main metabolic pathways that perform duties for modifying the gut microbiota. First, competing microbes for resources and available space inhibits the growth of dangerous bacteria, hence reducing their potential for pathogenicity (Bron et. al., 2017). Additionally, they may directly affect other bacteria, preventing pathogen adhesion and GI tract proliferation (Hemarajata, Versalovic, 2013). The synthesis of antimicrobial substances such as bactericides, organic acids, and short-chain fatty acids is involved in the second strategy (Cleusix et. al., 2007). Probiotics also stop potentially dangerous bacteria from growing in the GI tract by creating a hostile environment that kills off pathogens and inactivates their toxins (Bergonzelli et. al., 2005). Probiotics' third role is boosting and managing the immune system, encompassing both targeted and non-targeted responses. The processes of T cell activation, generation of cytokines, phagocytosis induction, and activation of IgA antibody discharge are used to achieve this (Corthésy, 2015) (Rescigno, Sabatino, 2009). Through their interactions with several cells in the immune system, probiotics cause the body to create lecithin, defensins, and antibacterial proteins. As a result, the body gets an advantage from altering the general composition of pro- and anti-inflammatory cytokines and from making it possible for potentially dangerous bacteria and/or their toxins to be inactivated (Everard et. al., 2014) (Cash et. al., 2006). One of the probiotics with the most research done on children and adults with functional gastrointestinal issues is *Lactobacillus reuteri* DSM 17938 (Dargenio et. al., 2022). For a probiotic strain to colonize, interact with host cells, reduce pathogens, protect epithelial cells, or modify immune responses, it must be

able to stick to the human GI tract (Lebeer et. al., 2008). Numerous investigations have confirmed the ability of *L. reuteri* to adhere to intestinal epithelial cells and to colonize mucus. The surface protein, mucus-binding protein (MacKenzie et. al., 2010), the antiadhesive characteristics of bacterial exopolysaccharides (reuteran and levan) (Walter et. al., 2008), the inulosucrase enzyme (Anwar et. al., 2012), D-alanyl-LTA (Walter et. al., 2007), and glucosyltransferase A (Athalye-Jape et .al., 2016) have all been connected to the potential mechanism behind adhesion. Furthermore, the antibacterial activity of *Lactobacillus reuteri* is one of the most extensively reported probiotic pathogen-inhibiting techniques. Lactic acid (Greifová et. al., 2017), acetic acid (Morita et. al., 2008), ethanol, reuterin, reutericyclin (Gänzle et. al., 2000), and histamine (Navarro et. al., 2017) are among the antimicrobial substances that *L. reuteri* produces. One of the best-known uses of *Lactobacillus reuteri* as a probiotic to cure illnesses is the treatment of *Helicobacter pylori* (Mukai et. al., 2002). According to Thomas et. al, 2012, histamine derived from *L. reuteri* 6475 inhibited the growth of tumour necrosis factor (TNF). This suppression required both the inhibition of MEK/ERK signaling and the activation of the histamine H2 receptor, which raised intracellular cAMP and protein kinase A (Thomas et. al., 2012). The results of this study suggest that *L. reuteri* may be useful in treating patients with gastrointestinal problems. Similarly, *L. reuteri* protected osteoporosis in an animal model of Type 1 diabetes, which is mediated by TNF-α and reduction of Wnt10b expression (Zhang et. al., 2015).

2.1.5 Relationship between *Lactobacillus reuteri* and the Gut-Brain Axis

In genetic, environmental, and idiopathic Autism Specific Disorders (ASD), *L. reuteri* has shown advancement in correcting the social deficiency(Sgritta et. al., 2019). The authors suggested that the gut-brain axis, in which *L. reuteri* affected GI function and gut microbiome, provided the basis for the physiological comprehension of *L. reuteri* based on a pilot investigation in which children with ASD received oral administration of 1010 colony-forming units of *L. reuteri* daily for a period of three months (Kong et. al., 2020). A lower amount of Lactobacillus spp. was seen in an animal model of autism that was genetically modified. Animals' unsocial behaviour was reduced when *L. reuteri* abundance was found, and there was a strong correlation found between the abundance of each of the three GABA receptor subunits and their expression (Tabouy et. al., 2018).

2.1.6 Role of *Lactobacillus reuteri* in Cystic Fibrosis

Dysbiosis, or alteration of the gut microbiota, has been associated with a reduction in beneficial bacteria and an overabundance of potentially dangerous bacteria in the gut of patients suffering from cystic fibrosis (Ooi, Durie, 2016). Probiotic therapy is becoming a more popular treatment

strategy for maintaining health and curing illnesses by altering the gut flora. When persons with cystic fibrosis take antibiotics on a regular basis for the treatment and prevention of pulmonary complications, probiotics may help restore the intestinal microbial equilibrium that has been upset (Gollwitzer, Marsland, 2014). Certain strains of *Lactobacillus reuteri* have been shown to have immunoregulatory effects and to help improve CF patients' gut health. Studies revealed that *L. reuteri* ATCC55730 was beneficial in reducing the frequency of infections associated with respiratory tract and pulmonary flare-ups in cystic fibrosis patients (Nardo et. al., 2014). It is now widely acknowledged that probiotics' principal impacts on the intestinal and extraintestinal tract are accomplished through their association with gut immunity, despite the long-held assumption that they may assist by reducing intestinal permeability (DuPont, DuPont, 2011) (Forsythe, 2011). Within this framework, a few researchers have postulated a gut-lung axis of probiotic activity, leading to the enhancement of the respiratory tract's innate and adaptive immune responses as a consequence of probiotic microorganisms stimulating the lymphoid tissues associated with the gut (Nardo et. al., 2014). The bronchial mucosa's elevated IgA-secretory cells, the proliferation of natural killer cells, also referred to as NK cells or large granular lymphocytes, the growth of T-regulatory cells, the generation of biologically active substances against lung infections, the suppression of factors that promote virulence, and the elevation of pulmonary macrophages' phagocytic activity could all be contributing factors to this gut-lung axis (Harata et. al., 2009) (Koizumi et. al., 2008) (Fink et. al., 2007). *L. reuteri* could potentially be able to alleviate the imbalance of the Cystic Fibrosis gut microbiota, which is characterized by a high concentration of proteobacterial species, according to another study. Furthermore, there was a substantial decline in inflammation in the intestine, a rise in microbial diversity, and a drop in the gut inflammatory marker "calprotectin" levels (Campo et. al., 2014). These findings thus corroborate the theory that specific *L. reuteri* strains affect the GI tract's immune response.

2.2 Experimentally explored physiological benefits of *Bacillus subtilis*

As mentioned earlier, apart from having high levels of resistance to both chemical and physical stimuli, in comparison to vegetative/active probiotics, spores are more resistant to technological stress and storage. Additionally, they are resilient to harsh intestinal and gastric conditions (pH, bile acids, and digestive enzymes) (Shinde et. al., 2019). Spore-forming probiotic bacteria are therefore seen to be an excellent alternative for strains of Lactobacillus and Bifidobacterium, which have the disadvantage of being less stable (Catinean et. al., 2019) (Jafari et. al., 2017). The benefits of utilizing Bacillus outlined above help to explain current initiatives to provide fresh insights into the application of spore-based probiotics, which show

stability comparable to other pharmaceuticals used in the traditional treatment of numerous illnesses (Foligné et. al., 2012).

2.2.1 Role of *Bacillus subtilis* in the synthesis of anti-microbial agents

The primary factor influencing Bacillus activity is its potential to synthesize antibiotics. The most efficient species is *B. subtilis*, which produces 66 antibiotics and dedicates 4%–5% of its genome to antibiotic synthesis. The antibacterial activity range and structure of Bacillus antibiotics vary (Reid et. al., 2003). Over the past 50 years, it has been known that *B. subtilis* has the ability to manufacture antibiotics (Caulier et. al., 2019). *B. subtilis* synthesizes both ribosomal synthesized peptides (Sumi et. al., 2015) and non-ribosomal synthesized peptides (Huang et. al., 2013) such as Subtilin, Ericin A and S, Mersacidin, Subtilosin A, Surfactin, Bacilysin, etc which have a huge spectrum of antimicrobial activity against gram-positive bacteria, viruses, mycoplasmas and several antibiotic-resistant strains too (Stein, 2005) (Xu et. al., 2012). Since these compounds are naturally occurring components of the human immune system, there is a decreased likelihood of pathogen resistance or undesirable side effects. Probiotic *B. subtilis* is therefore the best choice for treatment due to its wide range of activity and quick and targeted pathogen-killing action (Sumi et. al., 2015). The probiotic qualities of *B. subtilis* are strain-specific (Olmos et. al., 2020). *B. subtilis* has also been used to isolate pectinolytic enzymes.

2.2.2 *Bacillus subtilis* and its immunomodulatory effects

B. subtilis increases immunity to infections by promoting both specific and nonspecific immunity. There is substantial evidence that ingestion of Bacillus spores boosts the immune system based on a variety of research conducted in people and animal models. *B. subtilis* spores elicit particular immune responses that are both humoral and cell-mediated (Reid et. al., 2003). The host's innate and adaptive immune responses are developed in large part by the relationship between *B. subtilis* spores and macrophages. Activation of macrophages is caused by *B. subtilis*, as numerous investigations have shown (Xu et. al., 2012). Activation of macrophages is caused by *B. subtilis*, as numerous investigations have shown (Zamora-Pineda et. al., 2023). The spores of *Bacillus subtilis* have the potential to modulate immune responses by inducing pro-inflammatory cytokines and imparting probiotic effects through activated macrophage functions. Furthermore, *B. subtilis* was deemed harmless because it did not appear to be cytotoxic to RAW 264.7 cells (Xu et. al., 2012) (Zhao et. al., 2021). Administration of *B. subtilis* spores increases the response to antibodies (IgG and IgA) and stimulates T lymphocyte proliferation, suggesting that *B. subtilis* spores may enhance humoral and cellular immunity in

mice (Zhao et. al., 2021). Commensal bacteria are crucial for both innate and acquired immunity, as well as for the growth of the gut-associated lymphoid tissue (GALT). Within the GI system, *B. subtilis* stimulates active lymphocyte infiltration. It has been demonstrated that administering *B. subtilis* to the appendix of germ-free rabbits promotes GALT formation. This data demonstrated the significance of Bacillus species for the promotion of a strong immunological response and the establishment of a robust gut-associated lymphoid system (GALT) (Huang et. al., 2008). In healthy participants, oral administration of *B. subtilis* spores resulted in dose-dependent increases in the production of activation markers in lymphocytes (Caruso et. al., 1993). Mice exposed to *B. subtilis* spores had a widespread antibody response to ovalbumin and tetanus toxoid fragment C. According to these findings, *B. subtilis* spores are a useful additive for the mucosa and systemic lining that can be employed to boost humoral immune defences (Barnes et. al., 2007).

2.2.3 Role of *Bacillus subtilis* in preventing intestinal inflammation and maintaining homeostasis

In the host's intestinal epithelium, quorum-sensing pentapeptide generated from *B. subtilis*, competence, and sporulation factor (CSF) stimulate important survival mechanisms such as p38 MAP kinase and protein kinase B (Akt). In addition, CSF triggers the production of heat shock proteins, which shield intestinal epithelial cells from damage and the impairment of barrier function and allow the body to sustain intestinal homeostasis (Fujiya et. al., 2007). The *B. subtilis* quorum sensing molecule CSF increased the survival rate of animals with fatal colitis and decreased intestinal inflammation-induced epithelium damage. This suggests that *B. subtilis* may be advantageous in the management of intestinal inflammation. *B. subtilis* is useful in treating necrotizing enterocolitis and inflammatory bowel disease (IBD), including Crohn's disease, ulcerative colitis, and colitis brought on by antibiotics (Okamoto et. al., 2012). *In vitro*, it has been demonstrated that the Bacillus species (*Bacillus subtilis*, *B. firmus*, *Bacillus megaterium*, and *B. pumilus*) may transform genotoxic substances into non-reactive byproducts (Hong HA et. al., 2005). In suckling mice infected with *Citrobacter rodentium* (a model for the traveller's diarrhoea pathogen enterotoxigenic *Escherichia coli*), which has been found to induce epithelial lesions, crypt hyperplasia, and fatalities, oral administration of *B. subtilis* spores was effective in reducing infection and enteropathy (Sanders et. al., 2003) (D'Arienzo et. al., 2006).

2.2.4 Role of *Bacillus subtilis* against Antibiotic-associated Diarrhoea

Numerous studies have shown that *B. subtilis* beneficially regulates the normal gut flora. When treating experimental infections in mice, 3 strains of *B. subtilis* proved to be effective against

virulent cultures of *E. Coli* and Campylobacter species, and it preserved the animals' normal microbiota while they were receiving antibiotic therapy (Sorokulova, 2008). One of the most significant microbes for the prevention and treatment of intestinal illnesses in humans is *B. subtilis* (Mazza, 1994). When treating acute diarrhoea, *B. subtilis* responded better than *lactobacilli* (Sorokulova, 2008). Patients suffering from acute intestinal infections were given *B. subtilis* as part of clinical research. The results demonstrated the strong healing impact of Bacillus probiotics, as evidenced by a rapid recovery of stool to normal, the alleviation of abdominal pain, and the reduction of intestinal dysbiosis. Probiotics from Bacillus were proven to be both safe and well-tolerated (Gracheva et. al., 1996). The effects of *B. subtilis* on the microbiota of the intestine in cases of acute gastrointestinal issues and dysbacteriosis in 53 neonates with perinatal ailments have also been studied. The newborn children's diarrhoea and dysbacteriosis responded well to treatment and prophylaxis, with no adverse effects seen (Slabospitskaia et. al., 1995). Over 1800 people have participated in 23 clinical trials for probiotic preparations featuring *B. subtilis* R0179 with different probiotics. It has been used as a co-adjuvant therapy with sulfasalazine and mesalazine to improve recuperation times in minor to moderately severe ulcerative colitis, as well as to enhance adherence with traditional triple therapy for *Helicobacter pylori* elimination. These uses include the amelioration of symptoms related to chronic diarrhoea and irritable bowel syndrome(Tompkins et. al., 2010).
Owing to the substantial medical advantages of probiotic supplements, the global market for them has expanded during the past 20 years. The most prevalent genera are Lactobacillus and Bifidobacterium, mostly because of their capacity to keep infections out. However, they lack *B. subtilis*'s multipurpose therapeutic capabilities. Scientists are becoming increasingly intrigued by Bacillus bacteria as potential probiotics because of their potent antimicrobial, antidiarrheal, and immunostimulatory attributes, as well as their capacity to promote the growth of natural flora, lower intestinal inflammation, and maintain a high degree of balance under adverse environmental conditions. Additionally, it has been confirmed and approved by regulatory bodies such as the FDA and EFSA due to its proven efficacy and safety in many randomized, double-blind clinical trials. Thus, *B. subtilis* may prove to be the "ideal multipurpose and the most versatile class of probiotic" for treating an array of physiological ailments in patients.

CHAPTER 3

MATERIALS AND METHODS

Contents

3. MATERIALS AND METHODS

3.1 Chemicals

Butanol, Sodium Azide, Pyridine, Glycerol, Hydrogen Peroxide Solution, Hydrochloric Acid Solution, and Bovine Serum Albumin (BSA) were procured from Sigma-Aldrich, MO, USA. Bioassay Kits were procured from ARKRAY Healthcare Private Limited, India to measure several biochemical parameters. *Lactobacillus* MRS Broth and Tryptone Soya Broth were obtained from Himedia Laboratories Private Limited in India. sodium arsenite, ethylenediaminetetraacetic acid (EDTA), Tris HCl, potassium dihydrogen phosphate, 5,5'-disthiobis-(2-nitrobenzoic acid), methanol, nitro blue tetrazolium (NBT), NADH, phenazine methosulphate, GSH, 2,4-dinitrophenylhydrazine, trichloroacetic acid, ethanol, dischlorodihydrofluorescein diacetate (DCFDA), sodium dodecyl sulphate, and thiobarbituric acid (TBA) were obtained from Sisco Research Laboratory, Mumbai, India. Every reagent utilized was of analytical grade.

3.2 Preparation of Bacterial Strain

Lactobacillus reuteri ATCC 23272 DSM 20016, used in this study, was obtained from the American Type Culture Collection (ATCC), The Global Bioresource Centre, USA. The frozen stock culture strains were grown overnight on de Man-Rogosa-Sharpe (MRS) broth containing 15 mM glucose at 37°C without shaking. *Bacillus subtilis* MTC 441 strains were also used in this study and they were procured from the Microbial Type Culture Collection (MTCC), IMTECH Chandigarh, India. All the test strains were cultured on nutrient agar at 35 ± 1 °C and maintained at 4 ± 1 °C. A single colony of both strains was then transferred to MRS Broth and grown at 37°C for 24 hours separately. Following this, the cells were inoculated in MRS Broth and then grown for another 24 hours. The cells were harvested by centrifugation at 8000×g for 3 min and aliquots of 10^8 Colony Forming Units (CFU) were prepared in Tryptone Soya Broth with 10% Glycerol and stored at -80°C. (Jiang et. al., 2021) Before administration, the cells from both strains were washed twice in sterile Phosphate Buffer Saline (PBS) and resuspended in 1ml of PBS. Each rat was then orally administered with 1ml of PBS containing 2×10^8 CFU of the probiotic bacterial strains.

3.3 Experimental Animals

Twenty-four Male Wistar rats of weight ranging between 180 and 200g were obtained from Saha Enterprise, Kolkata, West Bengal, India. The rats were kept in standard polypropylene cages of dimensions 29×22×14cm. Animals were maintained under standard laboratory

conditions of temperature (20 ± 2 ºC), relative humidity (50 ± 15%), 12 h light-dark cycle, standard diet, and water ad libitum and all of these was carried out in the animal house of the Department of Pharmaceutical Technology at Jadavpur University, Kolkata. Instructions prescribed by our Institutional Animal Ethics Committee (IAEC) (Approval number JU/IAEC-22/35) were followed throughout the experiment. All the experimental procedures were performed, carefully keeping in mind and by abiding by the Principles of Laboratory Animal Care (Public Health Service, 2015). An acclimatization period of 14 days was observed before starting the *in vivo* experiment.

3.4 ***In vivo*** **Bioassay**

3.4.1 **Experimental setup**

The *in vivo* experiment was performed as per the established protocol in our laboratory (Das et. al., 2010). Twenty-four Male Wistar Rats (♂) were divided into 6 groups(n=6) and treated as mentioned below:

Group I: In the control group, rats were fed with 1ml Phosphate Buffer Saline (PBS) via an intragastric tube once a day for 10 days;

Group II: In the toxic control group, rats were treated with sodium arsenite (10mg/kg body weight, p.o., once daily) via an intragastric tube once a day for 10 days;

Group III: In the first treatment group, the rats received a daily dose of sodium arsenite at a concentration of 10mg/kg body weight, along with 1mL of *Lactobacillus reuteri* at a concentration of 2×10^8 CFU/mL administered orally via an intragastric tube, once a day, for 10 days.

Group IV: In the second treatment group, the rats received a daily dose of sodium arsenite at a concentration of 10mg/kg body weight, along with 1mL of *Bacillus subtilis* at a concentration of 2×10^8 CFU/mL administered orally via an intragastric tube, once a day, for 10 days.

The dosage of *Lactobacillus reuteri* and *Bacillus subtilis* was calculated after a thorough comprehension of the literature available about the therapeutic and protective actions these probiotic strains have shown (Das et. al., 2010). The intake of food and water was monitored twice a day. After 10 days, at the end of this experiment, rats were fasted overnight for around 16 hours, and on the 11^{th} day, they were sacrificed under euthanasia. Before sacrificing the rats, their body weight was also calculated. For the measurement of

biochemical markers, the blood samples were obtained from the retro-orbital venous plexus after applying tetracaine (0.5%) to the eyes (Das et. al., 2018). The livers were excised and cleaned with PBS. The weight of each liver was recorded. The liver was then divided into 2 portions, one was preserved in 10% formalin solution for further histological investigations while the other was immediately homogenized in Tris–HCl (0.01 M) + EDTA (0.001 M) buffers of pH 7.4 and centrifuged (12,000g) at 4°C for 30 min to obtain tissue homogenate. A schematic overview of the *in vivo* experimental setup is presented below:

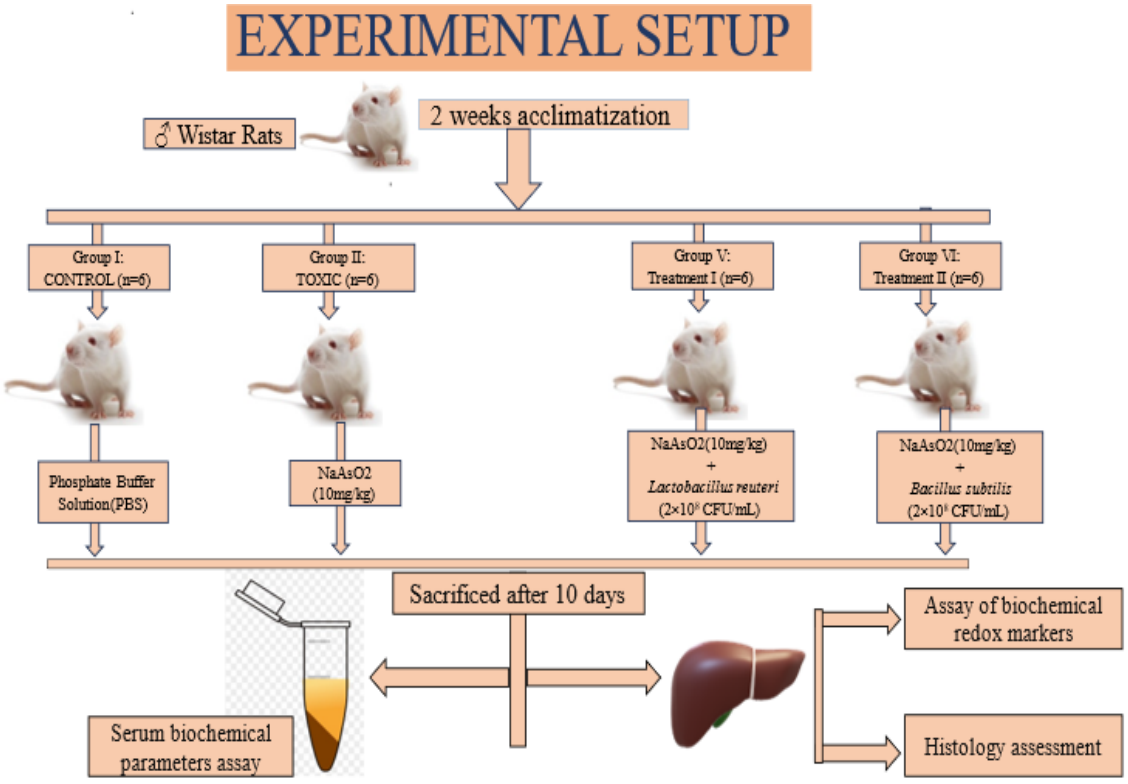

Figure 1. Schematic overview of the experimental protocol

3.4.2 **Estimation of Serum Biochemical Parameters**

Using commercially available kits (ARKRAY Healthcare Private Limited, India), the levels of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), Creatinine Kinase (CK), and C-reactive protein (CRP) in the sera were measured in accordance with the instructions provided in the manual by the provider.

3.4.3 **Assays for Biochemical and Redox Markers in Liver**

The levels of ROS, GSH, SOD, CAT, lipid peroxidation, and protein carbonylation in the livers of the rats that received different probiotic treatments were measured according to established protocols (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 established protocols (Manna et. al., 2022).

3.4.4 **Measurement of *in-vitro* Arsenic Uptake**

The capacity of the probiotic strains *Lactobacillus reuteri* and *Bacillus subtilis* to uptake different concentrations of Arsenic in different time frames under *in vitro* conditions also mentioned as Biosorption in several works of literature, was measured in UV Double Beam Spectrophotometer (Lab India model number 3200, India) explained in the referred protocol (Yan et. al., 2010).

3.4.5 **Histological Studies**

To conduct the histological analysis of the liver tissue, a portion of it from experimental animals during sacrifice, was carefully washed with ice-cold phosphate buffer solution at a pH of 7.4. Later, the tissue was preserved in a 10% formaldehyde solution and subsequently mounted in paraffin blocks for sectioning. In accordance with the established protocol, the sections were appropriately stained using haematoxylin and eosin(H&E) stains and imaged using a Leica DFC 450C microscope at a magnification of 100X (Dewanjee et. al., 2013).

3.4.6 **Statistical Analysis**

Experiments were performed in triplicate. The mean ±SD values were used to represent the data obtained from the experiment. The results underwent statistical analysis using one-way ANOVA followed by Dunnett's t-test with the assistance of GraphPad InStat (version 3.05), GraphPad Software, USA. Any p-values below 0.01-0.05 were deemed significant.

CHAPTER 4

RESULTS AND DISCUSSION

Contents

4. RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Effect of *L. reuteri* and *B. subtilis* on sodium arsenite intoxication

4.1.1.1 Impact on body weight and liver weight of the rats

No major change in the food and water requirements of either of the experimental groups was observed throughout the experiment. However, a decrease in the body weight of rats treated with sodium arsenite was noticed. But when they were given *L. reuteri* and *B. subtilis*, they regained weight up to some extent. A reverse pattern was observed in Liver weight. A significant increase was observed in rats intoxicated with sodium arsenite as compared to the ones given normal food and water i.e., the control group(Group I). However, the other 2 groups treated with *L. reuteri* and *B. subtilis*, had their liver weights similar to the control group.

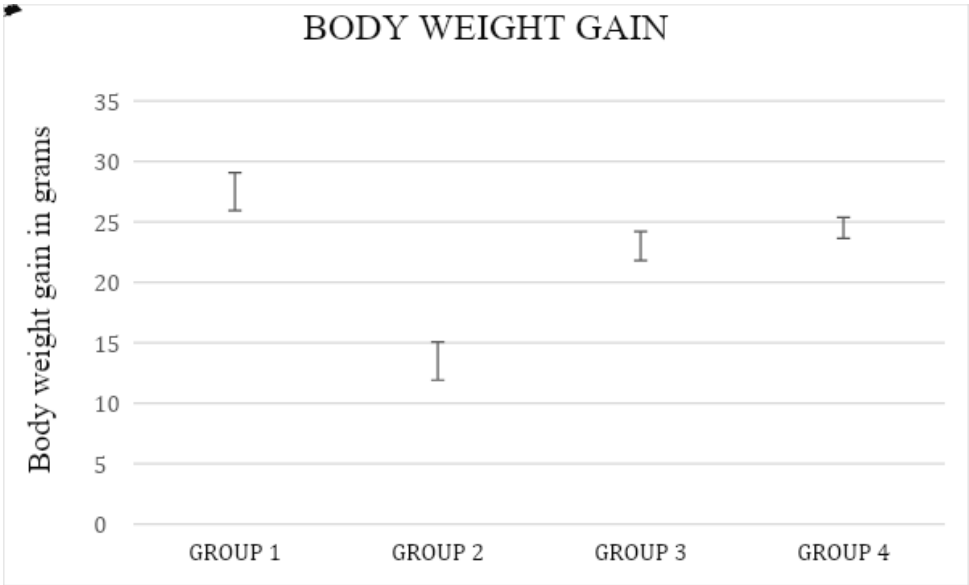

Figure 2. Impact of sodium arsenite on body weight gain in experimental rats. Values are expressed as mean±SD (n=6).

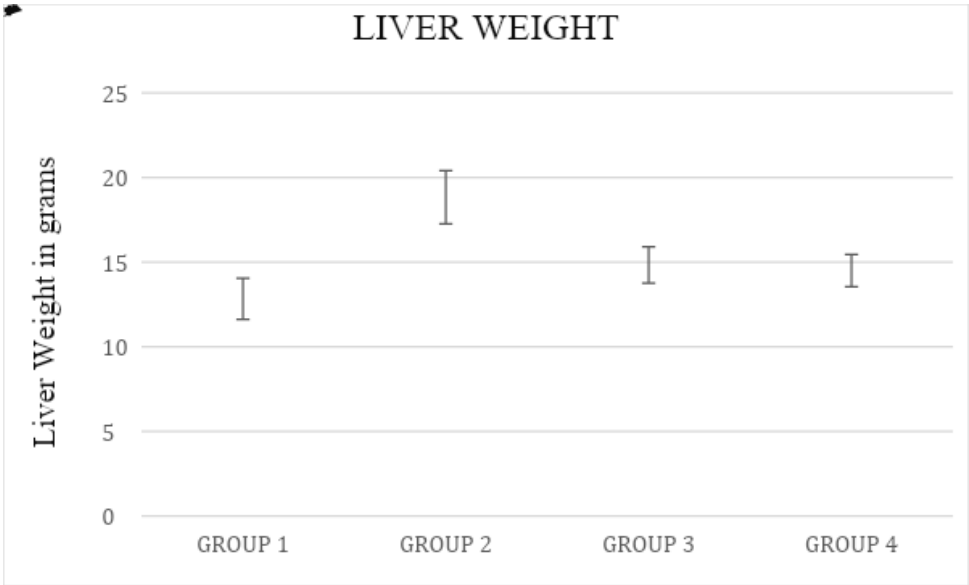

Figure 3. Impact of sodium arsenite Intoxication on the liver of rats. Values are expressed as mean±SD (n=6).

4.1.1.2 Effect on Serum Biochemical Parameters

Sodium arsenite intoxication damaged hepatocytes which caused enzymes like ALT, AST, and ALP to leak into the bloodstream. Their elevated concentrations increase hepatocellular damage. Treatment with *L. reuteri* and *B. subtilis* controlled the damage and put the enzymatic level nearly back to the control levels, which indicates their beneficial effects on hepatotoxicity. The effects of sodium arsenite intoxication on serum biochemical parameters are demonstrated by the significant rise of ALT, AST, ALP, LDH, CRP, and CK in comparison with the control group but co-administration of *L. reuteri* and *B. subtilis* reduced all of these to near normal state. The effects of sodium arsenite and treatment with *L. reuteri* and *B. subtilis* are shown in the **Figure 4** given below.

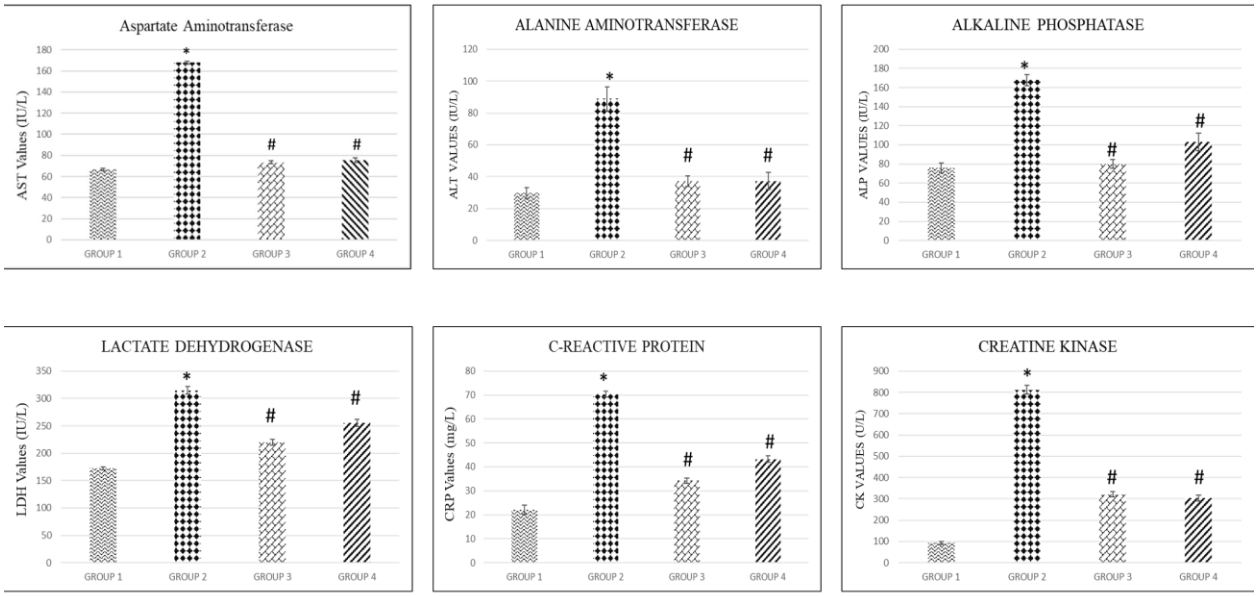

Figure 4. Effect of sodium arsenite intoxication and treatment with *L. reuteri* and *B. subtilis* on serum biochemical parameters in the experimental rats. Values are expressed as mean±SD (n=6). ***** indicates $p<0.01$ with respect to the control group (Group I); **#** indicates $p<0.01$ with respect to toxic group(Group II)

4.1.1.3 Effect of Redox Markers in Liver

The rats intoxicated with sodium arsenite exhibited an increase in the intracellular levels of ROS, lipid peroxidation (TBARS level), and protein carbonylation, within the hepatic tissue. However, the rats that were treated with *L. reuteri* and *B. subtilis* along with the co-administration of sodium arsenite, had these redox markers significantly reduced as compared to the ones that were not given any treatment. On the contrary, sodium arsenite reduced GSH levels and endogenous antioxidant enzymes like SOD and CAT in the hepatic tissue, further worsening the condition of the liver. However, simultaneous administration of *L. reuteri* and *B. subtilis* effectively restored and brought the GSH levels and antioxidant enzymes back to normal up to a great extent. **Figure 5** below shows the effect of *L. reuteri* and *B. subtilis* on sodium arsenite-intoxicated experimental rats.

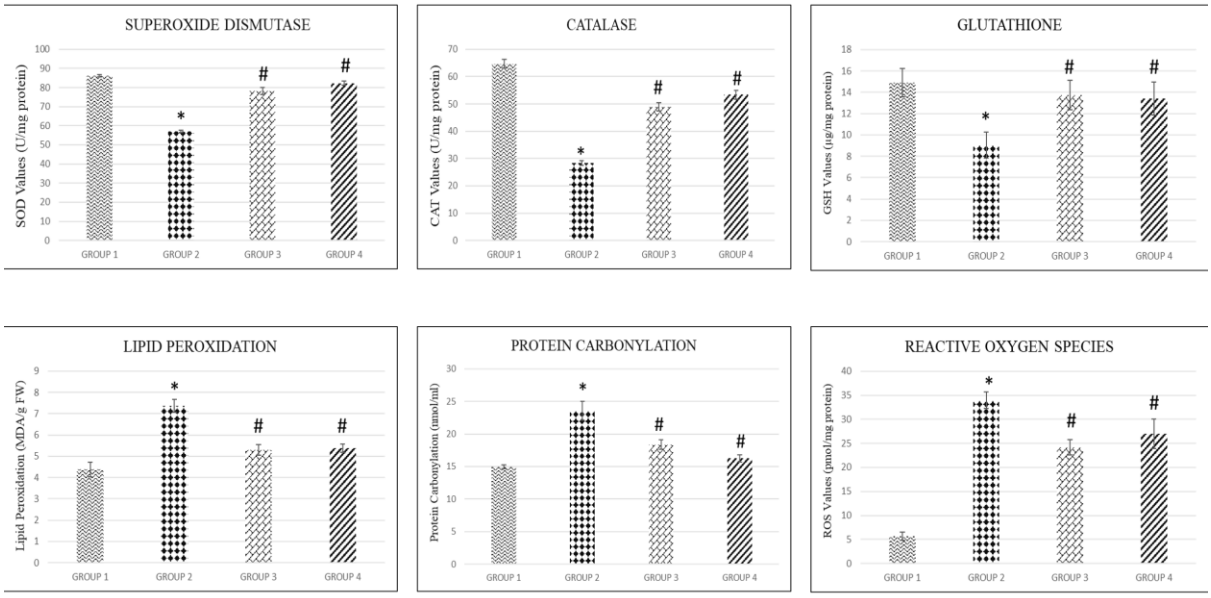

Figure 5. Effect of sodium arsenite intoxication and treatment with *L. reuteri* and *B. subtilis* on redox markers in the liver tissue of the experimental rats. Values are expressed as mean±SD (n=6). ***** indicates $p<0.01$ with respect to the control group(Group I); **#** indicates $p<0.01$ with respect to toxic group(Group II)

4.1.1.4 Effect of *L. reuteri* and *B. subtilis* on in-vitro concentrations of Arsenic

The results indicated that when compared simultaneously, *L. reuteri* performed better than *B. subtilis* in up taking Arsenic with a contact time ranging from 0 mins to 180 mins at both concentrations i.e., 500mg/L sodium arsenite and 1000mg/L sodium arsenite. **Figure 6** and **Figure 7** below show the uptake capacity of both strains under constant temperature and pressure conditions.

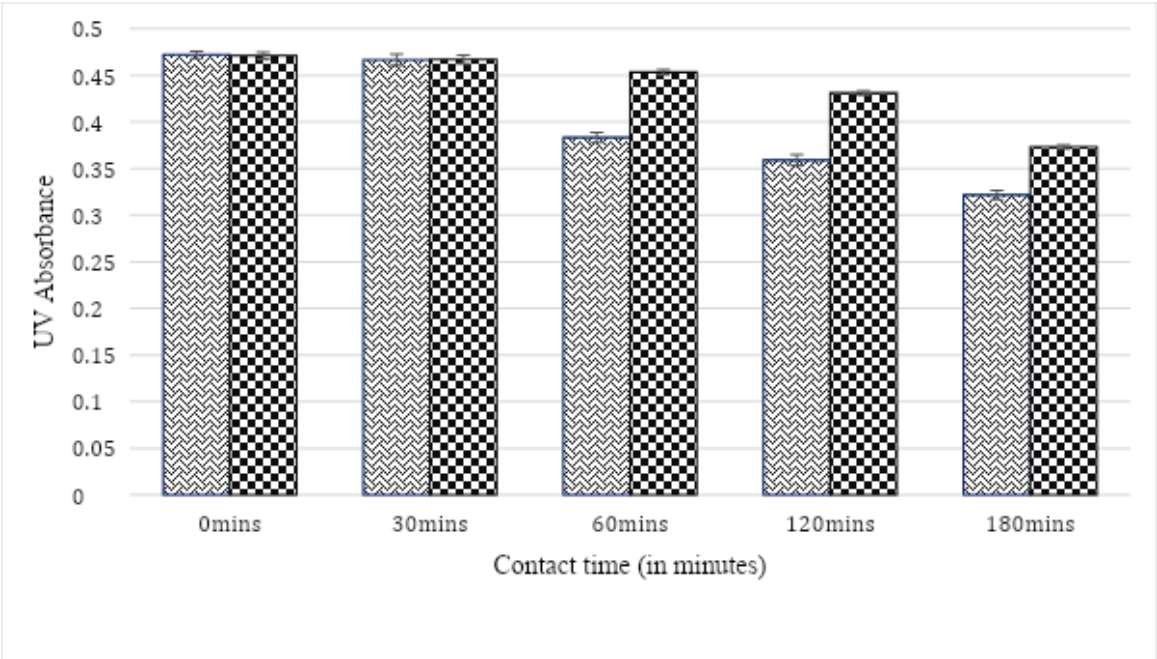

Figure 6. *In vitro* Arsenic uptake by ***L. reuteri*(Series 1)** and ***B. subtilis*(Series 2)** at 500mg/L sodium arsenite concentration. Values are expressed as mean±SD (n=3).

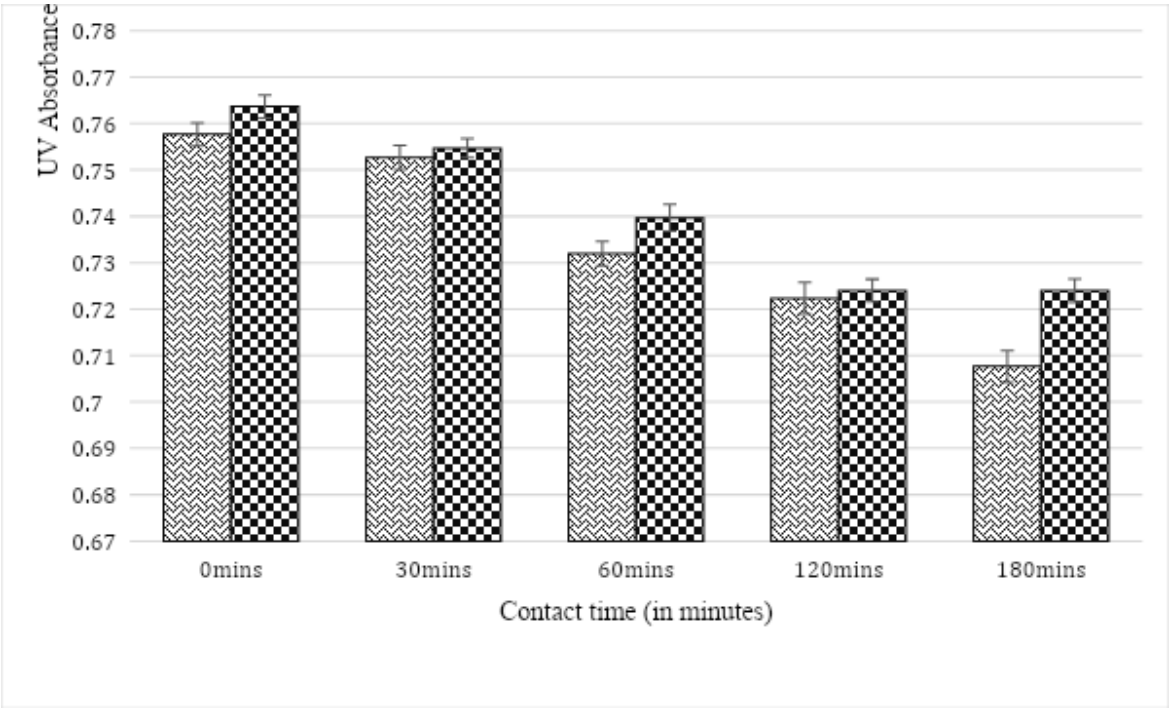

Figure 7. *In vitro* Arsenic uptake by ***L. reuteri*(Series 1)** and ***B. subtilis*(Series 2)** at 1000mg/L sodium arsenite concentration. Values are expressed as mean±SD (n=3).

4.1.1.5 Effects on Histology of Hepatic Tissue

Histological sections (100X) of the experimental animals are given in **Figure 8**. It provides a brief idea of the hepatic injury caused by sodium arsenite intoxication and the ameliorative effects when treated with *L. reuteri* and *B. subtilis.* The images in the figure strengthen the notion of the hepatoprotective effects of *L. reuteri* and *B. subtilis* against arsenic-induced hepatotoxicity.

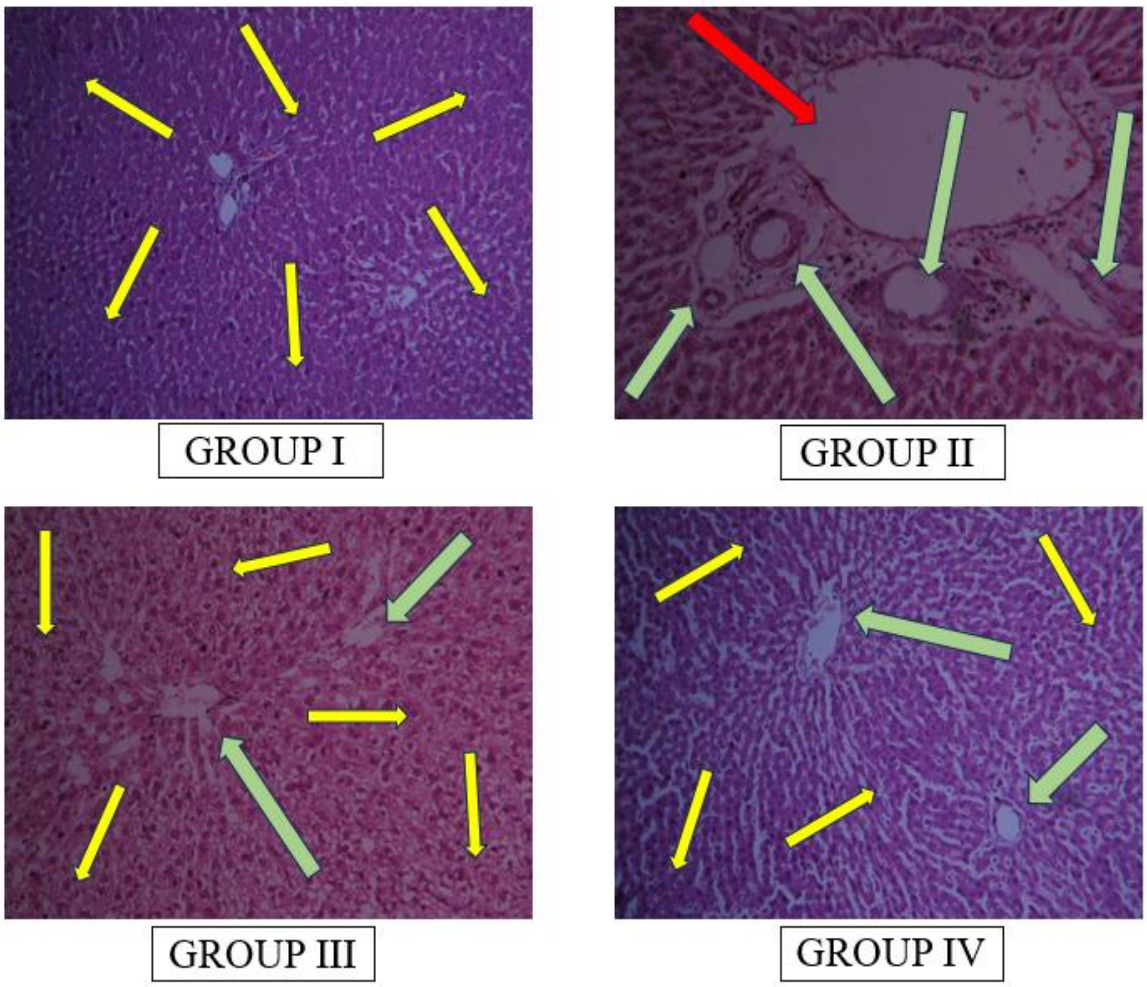

Figure 8. Haematoxylin-eosin (H&E) stained histopathological observations of rat liver. The hepatic tissue section of a normal rat(Group I) exhibited normal and intact hepatocytes (shown by yellow arrows). The liver of rats exposed to sodium arsenite(Group II) showed a dilated portal vein (indicated by red arrow) and had several apoptotic anomalies (indicated by green arrows) as compared to the control group. However, the livers of the sodium arsenite-intoxicated group of rats which were co-administered with *L. reuteri* (Group III) and *B. subtilis* (Group IV) indicate a possible reversal of the hepatocellular damage caused by sodium arsenite(shown by green arrows) and the hepatocytes seem to re-attain their

normal morphology (indicated by yellow arrows) when compared to the control group (Group I).

4.2 Discussions

Arsenic, a naturally occurring metalloid, is known for its toxic effects on various organ systems, with the liver being a primary target (Mirza et. al., 2014). Hepatotoxicity, or liver damage caused by poisonous substances, due to arsenic exposure is a significant concern, especially in areas where drinking water is contaminated with arsenic. Arsenic poisoning from drinking water contamination is an enormous problem that affects everyone around the globe. The hepatotoxic effects of arsenic are complex and involve multiple mechanisms, which can lead to both acute and chronic liver injury. Mechanisms of Arsenic-Induced Hepatotoxicity involve oxidative stress (Lantz and Hays, 2006).. Arsenic exposure leads to the generation of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals. These ROS are highly reactive and can damage cellular components, including lipids, proteins, and DNA (Flora, 2011). These ROS can further initiate the process of lipid peroxidation, where free radicals attack lipids in cell membranes, leading to membrane damage, increased membrane permeability, and ultimately cell death. In the liver, this can compromise hepatocyte integrity, leading to cell injury and apoptosis. Arsenic interferes with the body's antioxidant defence systems by depleting glutathione (GSH) and impairing the function of antioxidant enzymes like superoxide dismutase (SOD) and catalase (Laborde, 2010). This exacerbates oxidative stress and enhances liver damage. Few studies also indicate that arsenic contamination also leads to mitochondrial dysfunction (Nemeti et. al., 2002). Arsenic disrupts mitochondrial function by inhibiting the electron transport chain (ETC), particularly complexes I and III. This leads to a decrease in ATP production and an increase in electron leakage, further contributing to ROS generation. Arsenic has also been related to inducing mitochondrial permeability transition, a process that causes the mitochondrial membrane to become permeable to small molecules. This can result in the loss of mitochondrial membrane potential, release of pro-apoptotic factors like cytochrome c, and activation of the intrinsic pathway of apoptosis in hepatocytes. Arsenic exposure can activate various pro-inflammatory signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs). These pathways lead to the production of inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukins (IL-1β, IL-6). Kupffer cells, the resident macrophages of

the liver, are activated in response to arsenic-induced oxidative stress and inflammation (Cui et. al., 2019). Activated Kupffer cells produce more ROS and release cytokines, perpetuating liver inflammation and injury. As a result of all the inflammation and depletion of anti-inflammatory markers, mechanisms of cell death also known as apoptosis and necrosis are triggered, worsening the situation even more. Arsenic-induced apoptosis is mediated by both the intrinsic (mitochondrial) and extrinsic (death receptor) pathways. The release of cytochrome c from mitochondria and the activation of caspases (such as caspase-3) are key events in this process. In cases of severe arsenic toxicity, the cellular damage can overwhelm the apoptotic machinery, leading to necrosis, a form of cell death characterized by cell swelling, membrane rupture, and inflammation. Necrosis further exacerbates liver damage and can lead to fibrosis over time. Not only cellular but arsenic also infiltrates the genetic level thus causing several epigenetic modifications. Arsenic can alter the methylation status of DNA, leading to changes in gene expression (Sharma et. al., 2014). Hypomethylation of oncogenes and hypermethylation of tumour suppressor genes have been observed in arsenic-exposed individuals, contributing to the risk of hepatocellular carcinoma. Arsenic exposure can also induce histone modifications, such as acetylation and methylation, which can affect chromatin structure and gene expression, leading to disrupted cellular functions and increased susceptibility to liver damage. Arsenic disrupts the hepatic metabolism by interfering with heme synthesis and degradation, leading to the accumulation of porphyrins and other intermediates that are toxic to hepatocytes. This disruption can also impair the detoxification processes in the liver, increasing vulnerability to further toxic results. Arsenic can disrupt the normal synthesis and secretion of bile acids, leading to cholestasis (bile flow obstruction) and hepatocyte injury. This can further contribute to the development of fibrosis and cirrhosis (Shi et. al., 2017). There are several clinical implications involved with arsenic toxicity. Some of these are symptoms of acute hepatitis, including jaundice, elevated liver enzymes (AST, ALT), and abdominal pain. Chronic exposure to lower levels of arsenic is associated with the development of liver fibrosis, cirrhosis, and an increased risk of hepatocellular carcinoma (HCC). Arsenic exposure has also been linked to the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), conditions characterized by fat accumulation in the liver, inflammation, and liver damage(Boursier et. al., 2016).

Although antioxidant therapy and chelation therapies are already available as treatment options, their effectiveness is not as high as it should be (Susan et. al., 2019). For this reason, we have investigated the potential of probiotic strains to mitigate arsenic toxicity.

Probiotic strains such as *Lactobacillus reuteri* and *Bacillus subtilis* have shown promise in reducing arsenic-induced hepatotoxicity through various protective mechanisms. These probiotics can mitigate liver damage caused by arsenic exposure by modulating gut microbiota, enhancing the body's detoxification processes, reducing oxidative stress, and modulating the immune response. Arsenic exposure can disrupt the gut microbiota, leading to dysbiosis, which is associated with increased intestinal permeability ("leaky gut") and systemic inflammation"(Paolella et. al., 2014). *Lactobacillus reuteri* and *Bacillus subtilis* help restore a healthy balance of gut bacteria, promoting the growth of beneficial microbes and inhibiting pathogenic ones. This balance is crucial in preventing the translocation of endotoxins and arsenic from the gut into the bloodstream, thereby reducing liver exposure to these harmful substances. These probiotics can bind to arsenic in the gut, reducing its absorption and promoting its excretion (Lo et. al., 2014). This reduces the overall arsenic burden in the body, leading to lower levels of arsenic reaching the liver and other organs. By reducing oxidative stress, these probiotics help protect hepatocytes from arsenic-induced lipid peroxidation and DNA damage. These probiotics can also upregulate the expression and activity of the body's antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Enhanced antioxidant defences help counteract the oxidative damage caused by arsenic exposure. As shown in **Figure 5**, *L. reuteri* and *B. subtilis* have substantially reduced ROS formation and increased the deterring levels of GSH, thus reversing the damage posed to the hepatocytes, which is evident in the histopathological images attached in **Figure 8**. Probiotics may enhance the body's natural detoxification pathways. For example, *Bacillus subtilis* is known to produce enzymes that can assist in detoxifying heavy metals and other toxins. This can help in reducing the hepatic load of arsenic. Some probiotic strains, including *Lactobacillus reuteri*, have metal-binding properties. They can chelate arsenic ions, thereby reducing their bioavailability and toxicity. This binding occurs in the gut, where the chelated arsenic is excreted through faeces, reducing the amount that reaches the liver. The findings of this investigation indicate that *L. reuteri* and *B. subtilis* offer several benefits when it comes to liver health, from reversing oxidative injury caused by Arsenic to maintaining antioxidant hepatic enzymes and redox markers. The results project that *L. reuteri* and *B. subtilis* can efficiently act against arsenic intoxication, without compromising the body weight of the individual, and by maintaining a healthy liver weight, shape, and morphology. These strains proved that they can actively maintain lipid peroxides, protein carbonyls, inflammatory redox markers, and the apoptosis of hepatocytes caused by arsenic. Not only that, these

strains can easily help the body recover from hepatic injury by recovering the various enzymes such as AST, ALT, ALP, LDH, CRP, and CK. The deterring impact of arsenic on the liver and the ameliorative effects of *L. reuteri* and *B. subtilis* was properly demonstrated by the Haematoxylin & Eosin-stained histopathological investigations.

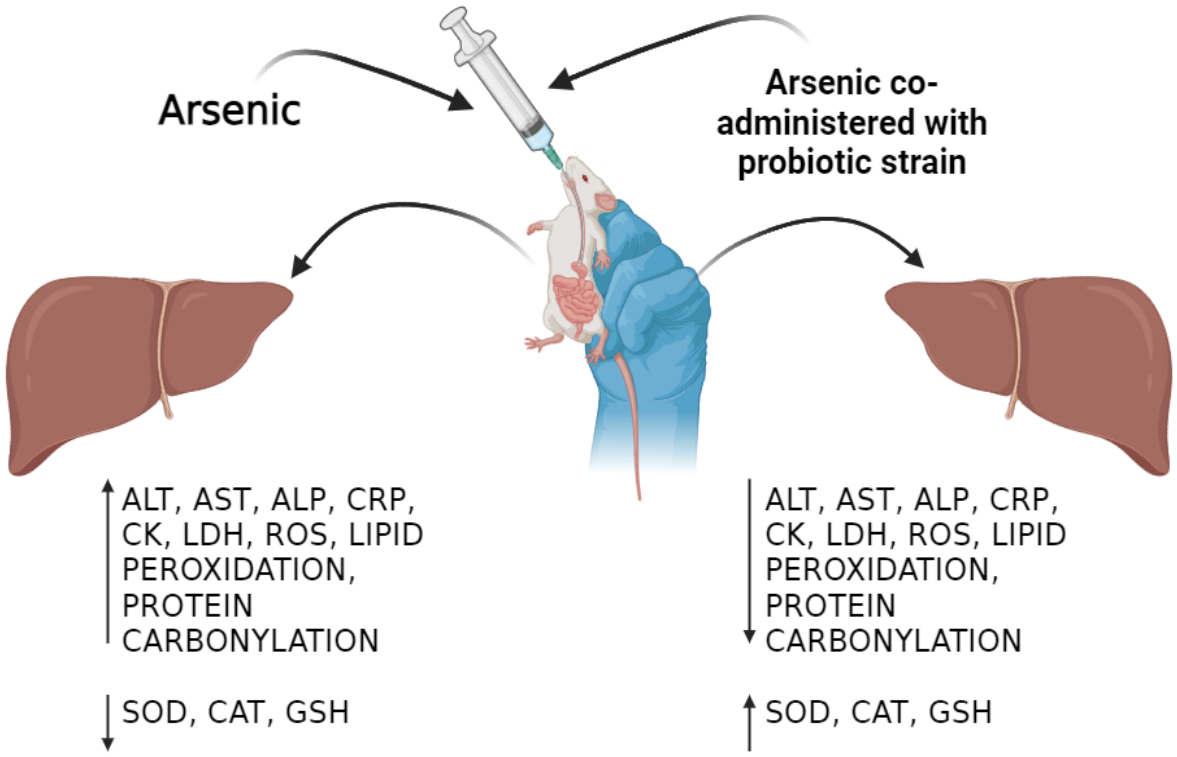

Figure 9. The deteriorative effects of Arsenic on Liver and the ability of *Lactobacillus reuteri* and *Bacillus subtilis* to ameliorate the harm.

CHAPTER 5

CONCLUSION

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This extensive research adds to a clearer understanding of the hepatoprotective effects of the probiotic strains *Lactobacillus reuteri* and *Bacillus subtilis* and offers substantial evidence of its intrinsic potential to be used as a treatment alternative for hepatic ailments. *Lactobacillus reuteri* and *Bacillus subtilis* reduce arsenic-induced hepatotoxicity through multiple mechanisms, including modulation of gut microbiota, enhancement of antioxidant defenses, promotion of detoxification processes, anti-inflammatory effects, and improvement of intestinal barrier function. These protective effects make these probiotics potential candidates for mitigating liver damage in individuals exposed to arsenic, especially in regions with high levels of environmental arsenic contamination. By their ability to significantly elevate the serum biochemical parameters such as AST, ALT, ALP, CK, CRP, and LDH, it is clearly evident that they can manage the hepatocellular damage caused by Arsenic toxicity. Along with that, employing this investigation, we also derived that *Lactobacillus reuteri* and *Bacillus subtilis* possess anti-oxidant properties as they restored the normal levels of ROS, lipid peroxidation, SOD, CAT, protein carbonylation, and reduced glutathione. This will prove advantageous, if, utilized in treating several oxidative stress-related disorders. These strains are exceptionally promising and should be studied more to explore more medical benefits that will eventually benefit mankind.

CHAPTER 6

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6. REFERENCES

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