MICROBIAL AND PATHOLOGICAL SCREENING OF *Lactobacillus casei* IN ENHANCING IMMUNITY AGAINST *Shigella flexneri* IN WISTAR ALBINO RATS

BY

DEBORAH NWABIANI CYRIL-ORIHU

LSC2009952

DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

BENIN CITY, EDO STATE

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL FULFILLMENT OF THE AWARD OF BACHELOR OF SCIENCE DEGREE (B.Sc HONS) IN MICROBIOLOGY.

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CERTIFICATION

This is to certify that this project work was carried out by Deborah Nwabiani Cyril-orihu in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under the supervision of Dr. (Mrs.) J.Z. Saidu.

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Dr. (Mrs.) J.Z. Saidu **Date**

(Supervisor)

APPROVAL

I certify that this work has been accepted in partial fulfillment of the requirement for the award of Bachelor of Science (B.Sc.) in the Department of Microbiology, University of Benin, Benin City.

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PROF. F. I. AKINNIBOSUN **Date**

(Head of Department)

DEDICATION

This project is dedicated to Almighty God and my supervisor Dr. (Mrs.) J.Z. Saidu and my mentor (Mr. Onaiwu Godspower).

ACKNOWLEGDEMENT

I would like to thank God for his continuous grace and guidance, for how good he has been to me throughout my schooling and for his protection throughout the research project. I would also like to express my sincere thanks to my supervisor Dr. (Mrs.) J.Z. Saidu and my sincere thanks to Mr. Onaiwu Godspower for his continuous guidance, support and knowledge impacted, it was really great working under you sir. I would like to thank my fellow students, friends and colleagues for the time we spent together and all the memories it brought. Special thanks to the head of department Prof. (Mrs.). F.I. Akinnibosun and other staff of the Department of Microbiology for their support.

ABSTRACT

Shigella flexneri, a leading cause of bacillary dysentery, induces severe gastrointestinal inflammation, disrupting gut homeostasis. This study evaluates the potential of *Lactobacillus casei* as probiotics to enhance immune responses in Wistar albino rats against *Shigella flexneri* infection. Forty healthy Wistar rats were divided into five groups: Control, challenged (*Shigella flexneri*-infected), Probiotic (*Lactobacillus casei*-treated), Prophylactic (pre-treated with *Lactobacillus casei* before infection), and Antibiotic (ciprofloxacin-treated). Stool samples were collected from rats prior to induction with *shigella flexneri* and *Lactobacillus casei*, after induction with *Shigella flexneri*, and antibiotic treatment. Approximately 1g of stool was homogenized in 9 mL of sterile PBS using a vortex mixer to ensure thorough dispersion. A tenfold serial dilution was performed by transferring 1 mL of the stool homogenate into 9 mL of sterile PBS, creating dilutions from 10' to 10-6. From the 1 dilution, 0.1 mL was plated onto Nutrient agar plates and incubated at 37°C for 24hrs. The Physical assessments of the wistar albino rats revealed that the temperature and stool in the control group were the same and with a slight increase in the weight of the rats while there was an increase in the temperature in the challenged group and a decrease in the rats weight. There was a decrease in temperature in the antibiotic group and increase in the rat weight and slight temperature increase the prophylactic group and increase in the weight. Histopathological analysis of the colon of the rats challenged with *Lactobacillus casei* and control group appeared normal, with no edema, no epithelial disruption, and no ulcerations or cellular infiltrations in the lamina propria. I recommend further research should be conducted to explore the long-term effects of Lactobacillus casei supplementation on immune function and gut microbiota and the potential use of Lactobacillus

casei as a probiotic supplement for managing enteric infections should be investigated in clinical settings.

CHAPTER ONE

INTRODUCTION

The role of *Lactobacillus* species in enhancing immunity against Shigella infection in rats has garnered significant attention in recent research. *Lactobacillus* species, a probiotic, have been shown to exert protective effects against various pathogens, including *Shigella*, through multiple mechanisms that enhance the host's immune response.

One of the primary mechanisms by which *Lactobacillus* species enhance immunity is through the modulation of gut microbiota. Studies have demonstrated that the administration of *Lactobacillus* can lead to a favorable shift in the gut microbiome, promoting the growth of beneficial bacteria while inhibiting pathogenic species such as *Shigella*. For instance, the combination of *Lactobacillus reuteri* and *Clostridium butyricum* has been shown to improve gut microbiota diversity and reduce the abundance of pathogenic bacteria, including those from the *Enterobacteriaceae* family, thereby enhancing the immune response in the host (*Hsiao et al*., 2021; Hu *et al*., 2022). Furthermore, *Lactobacillus* species can produce short-chain fatty acids which are known to have anti-inflammatory properties and can bolster the immune system by promoting the production of regulatory T cells (Sherman *et al*., 2022).

In experimental models, Lactobacillus strains have demonstrated direct antibacterial activity against *Shigella dysenteriae*. For example, *Lactobacillus lactis* and *Lactobacillus brevis* exhibited significant zones of inhibition against this pathogen, indicating their potential as therapeutic agents in preventing Shigella infections (Ibejekwe, 2023). Additionally, *Lactobacillus* species have been shown to enhance the overall health of the intestinal mucosa, which is crucial for maintaining an effective barrier against infections (Wang *et al*., 2021). Wang *et al*., (2021), reported that probiotics can stimulate immune cells, such as monocytes and

macrophages, thereby promoting immune homeostasis and enhancing the host's ability to combat infections. Moreover, the administration of Lactobacillus has been associated with improved clinical outcomes in infected rats. For instance, probiotics have been linked to normalizing liver function and reducing inflammatory markers in pathogen-infected rats, suggesting a systemic effect on health and immunity (Amengialue *et al*., 2023). The presence of *Lactobacillus* not only aids in the direct inhibition of pathogens but also contributes to the overall resilience of the host's immune system against infections (Pan, 2022). *Lactobacillus casei* competes with *Shigella flexneri* for nutrients and adhesion sites on the intestinal epithelium, preventing the pathogen from colonizing the gut. This reduces the ability of *S. flexneri* to establish infection, limiting its proliferation. *L. casei* produces lactic acid and bacteriocins, which create an unfavorable environment for *S. flexneri* (Kim *et al*., 2021). *Lactobacillus casei* also stimulates mucosal immunity, boosting the secretion of IgA antibodies, which help neutralize *S. flexneri* before it invades intestinal cells.

1.2 Aim and Objectives

The aim of this study is to investigates the potential of *Lactobacillus casei* as probiotics to enhance immune responses in Wistar albino rats against *Shigella flexneri* infection

The objectives of this research were to;

1. identify and enumerate *Lactobacillus casei* and *Shigella flexneri*
2. to investigate immune-modulatory effects of *Lactobacillus casei*
3. to evaluate the ability *Lactobacillus casei* to reduce the severity of *Shigella flexneri* infection in Wistar albino rats.

4. to investigate the microbial load from stool and organ samples of Wistar albino rats challenge with *Lactobacillus casei* and *Shigella flexneri*

5. to evaluate the potential of *Lactobacillus casei* as prophylactic and in comparison, to commercially purchased antibiotics

CHAPTER TWO

LITERATURE REVIEW

2.1 The Genus *Lactobacillus*

The genus Lactobacillus is a critical component of lactic acid bacteria (LAB), known for its diverse roles in fermentation processes across various food products. Lactobacillus species are primarily characterized as Gram-positive, non-spore-forming, catalase-negative rods that exhibit facultative anaerobic or microaerophilic growth conditions (Xie *et al*., 2019). This genus is notable for its ability to ferment carbohydrates into lactic acid, which is a key factor in food preservation and flavor development (Wang *et al*., 2021). Phylogenetically, Lactobacillus encompasses a variety of species that can be classified into two main categories based on their fermentation pathways: homofermentative and heterofermentative (Xie *et al*., 2019). Homofermentative species, such as *Lactobacillus delbrueckii* primarily convert glucose into lactic acid without producing gas, while heterofermentative species, like *Lactobacillus brevis*, can produce both lactic acid and other compounds, including carbon dioxide and ethanol (Zheng *et al*., 2015). The evolutionary relationships among Lactobacillus species and related genera, such as *Pediococcus* and *Leuconostoc*, have been elucidated through comparative genomic analyses, which indicate that these groups share a common phylogenetic lineage (Sun *et al*., 2015). Lactobacillus species are widely utilized in the fermentation of various foods, including dairy products, vegetables, and alcoholic beverages. *Lactobacillus plantarum* and *Lactobacillus casei* are frequently isolated from fermented vegetables like kimchi and pickles, where they contribute to the characteristic flavors and preservation of these products (Cho *et al*., 2006; Peng *et al*., 2018). In dairy fermentation, Lactobacillus species are essential for yogurt production, where they influence the texture, acidity, and flavor profile of the final product (Vénica *et al*.,

2022). Additionally, Lactobacillus is involved in the fermentation of alcoholic beverages, such as Chinese liquor, where it plays a role in flavor development through the production of various metabolites (Du *et al*., 2020). The ecological adaptability of Lactobacillus is also noteworthy. These bacteria can thrive in a range of environmental conditions, including varying pH levels and temperatures, which allows them to dominate in diverse fermentation environments (Song *et al*., 2017; Zhao *et al*., 2018). Their metabolic versatility enables them to utilize different substrates, making them integral to the fermentation processes of both plant and animal-derived foods (Peng *et al*., 2018; Wang, 2023). Furthermore, the presence of Lactobacillus in the gastrointestinal tract of humans and animals underscores their importance not only in food production but also in health, as they contribute to gut microbiota balance and may offer probiotic benefits (Naser *et al*., 2007; Elfahri *et al*., 2016).

2.2 Lactobacillus as Probiotics

Lactobacillus species are widely recognized as important probiotics, contributing significantly to human health through various mechanisms. Probiotics, defined as live microorganisms that confer health benefits when administered in adequate amounts, play a crucial role in maintaining gut health, enhancing immune function, and preventing various diseases (Rahimi and Himmat, 2022). Among the *Lactobacillus* genus, strains such as *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* have been extensively studied for their probiotic properties and therapeutic potential (Pino *et al*., 2019; Yılmaz & Şimşek, 2020). The health benefits of Lactobacillus probiotics are multifaceted. They are known to enhance the gut microbiota composition, which can improve digestion and nutrient absorption (Osita *et al*., 2019; Dempsey and Corr, 2022). Furthermore, Lactobacillus strains exhibit antimicrobial properties, which help inhibit the growth of pathogenic bacteria in the gastrointestinal tract. Certain

Lactobacillus strains produce bacteriocins and organic acids that lower the pH of the gut environment, making it less hospitable for harmful microorganisms (Prabhurajeshwar and Chandrakanth, 2016; Song *et al*., 2015). Additionally, these probiotics can modulate the immune response by interacting with immune cells and promoting the production of beneficial cytokines (C, 2015; Evrard *et al*., 2011).

Immunomodulatory effect is particularly significant in conditions such as inflammatory bowel disease and allergies, where a balanced immune response is crucial (Evrard *et al*., 2011; Dempsey and Corr, 2022). Furthermore, *Lactobacillus* as probiotics have been shown to provide benefits beyond gut health. For example, specific strains have been linked to improvements in vaginal health by maintaining a balanced microbiota and preventing infections (Pino *et al*., 2019; Li and So, 2021). The ability of Lactobacillus to adhere to mucosal surfaces enhances their effectiveness in colonizing the gut and vaginal microbiota, thereby exerting their beneficial effects (Osita *et al*., 2019; Li and So, 2021). Additionally, Lactobacillus strains have been implicated in the prevention of antibiotic-associated diarrhea and the management of lactose intolerance, showcasing their versatility as therapeutic (Amengialue, *et al*., 2023).

The efficacy of *Lactobacillus* as probiotics is strain-specific, meaning that not all strains confer the same health benefits. This specificity necessitates careful selection and characterization of probiotic strains for clinical applications (McFarland *et al*., 2018; Song *et al*., 2015). For instance, *Lactobacillus rhamnosus* has been extensively studied and is recognized for its effectiveness in preventing *Clostridium difficile* infections, while other strains may be more effective in different contexts (Prabhurajeshwar and Chandrakanth, 2016; McFarland *et al*., 2018). Therefore, ongoing research is essential to elucidate the mechanisms of action and health benefits associated with various *Lactobacillus* strains, ensuring their optimal use in health interventions.

2.3 Shigella Infections

Shigella infection, primarily caused by the species Shigella flexneri and *Shigella sonnei*, remains a significant public health concern globally, particularly in developing regions. The transmission of Shigella occurs predominantly through the fecal-oral route, often exacerbated by poor sanitation and contaminated water sources. *Shigella flexneri* is the most prevalent species in Asia, accounting for approximately 49% of reported cases, while Shigella *sonnei* follows closely with 43% (Salleh *et al*., 2022). The global burden of Shigella infections is substantial, with an estimated 165 million cases and 1.1 million deaths annually (Azmi *et al*., 2014).

The antibiotic resistance patterns of Shigella species have become increasingly alarming. A systematic review highlighted that a significant proportion of Shigella isolates exhibit resistance to commonly used antibiotics, including ampicillin and trimethoprim-sulfamethoxazole, with resistance rates reported as high as 95.2% for ampicillin in some regions (Salleh *et al*., 2022; Teslim, 2024). This resistance is often attributed to the misuse and overuse of antibiotics in treating diarrheal diseases, leading to the emergence of multidrug-resistant (MDR) strains (Farahani *et al*., 2018; Majalan *et al*., 2018). Jafari-Sales and Shariat, (2021), reported that *Shigella flexneri* isolates in Iran demonstrated high resistance rates to ampicillin (67.86%) and cotrimoxazole (92.85%). The presence of extended-spectrum beta-lactamase (ESBL)-producing strains further complicates treatment options, as these strains exhibit resistance to third-generation cephalosporins (Dhital *et al*., 2017; Nisa *et al*., 2022). The development of antibiotic resistance in Shigella is facilitated by genetic elements such as plasmids and transposons, which can transfer resistance genes between bacteria (Aluntaş *et al*., 2018). This genetic adaptability underscores the need for vigilant monitoring of resistance patterns and the implementation of effective infection control measures. Furthermore, the environmental persistence of Shigella in

contaminated water sources poses additional challenges, as evidenced by studies demonstrating the efficacy of bacteriophages in controlling Shigella in water (Jun *et al*., 2016).

2.4 Pathogenicity of *Shigella flexneri*

Shigella flexneri is a significant entero-invasive pathogen responsible for bacillary dysentery, or shigellosis, primarily affecting children in developing countries. Its pathogenicity is characterized by a complex interplay of virulence factors, including the type III secretion system (T3SS), which facilitates the injection of effector proteins into host cells, leading to cellular alterations that promote bacterial invasion and survival.

The T3SS is crucial for the virulence of S. flexneri, as it allows the bacterium to manipulate host cell processes. For instance, the effector proteins secreted through this system can induce cytoskeletal rearrangements, facilitating bacterial entry into epithelial cells (Lum *et al*., 2013; Mazon-Moya *et al*., 2017). Once inside, *S. flexneri* can escape from the phagosome into the cytosol, where it replicates and spreads to adjacent cells, a process that is critical for its pathogenicity (Mostowy *et al*., 2013; Koestler *et al*., 2019). The ability of S. flexneri to evade the host immune response is further enhanced by its capacity to induce apoptosis in immune cells, such as B lymphocytes, thereby undermining the host's defense mechanisms (Nothelfer *et al*., 2014).

Another aspect of *S. flexneri* pathogenicity is its iron acquisition mechanisms. The bacterium utilizes siderophores to scavenge iron from the host, which is essential for its growth and survival within the host environment (Luck *et al*., 2001; Walker and Verma, 2002). The presence of pathogenicity islands, such as SHI-1 and SHI-2, contributes to the genetic basis of its virulence, encoding various factors that enhance its ability to cause disease (Yang, 2005). These islands not only harbor genes for iron acquisition but also other virulence determinants that

facilitate the bacterium's adaptation to the host. The interaction of S. flexneri with host cells is also influenced by its ability to induce inflammation and modulate immune responses. The bacterium can manipulate signaling pathways within the host, leading to altered immune responses that favor its survival (Burton, 2003). For example, the activation of specific kinases during infection can promote bacterial entry and replication, highlighting the sophisticated strategies employed by *S. flexneri* to establish infection (Burton, 2003).

2.5 Lactobacillus as Immune Enhancer Against Shigella Infection

Lactobacillus species have been recognized for their potential role as immune enhancers against Shigella infections. The mechanisms through which Lactobacillus exerts its protective effects are multifaceted, involving modulation of the gut microbiota, production of antimicrobial substances, and enhancement of immune responses.

Research indicated that Lactobacillus can inhibit the growth of Shigella through various mechanisms, including the production of organic acids and hydrogen peroxide, which lower the pH and create an unfavorable environment for pathogenic bacteria (Mirzaei *et al*., 2018). Furthermore, Yang et al. demonstrated that the presence of Lactobacillus in the gut microbiota can significantly reduce the abundance of Shigella, suggesting a competitive exclusion mechanism (Yang *et al*., 2020). This is particularly relevant given the rapid changes in gut microbiota composition following Shigella infection, where probiotics like Lactobacillus can help restore balance (Yang *et al*., 2020). In addition to direct antimicrobial activity, Lactobacillus is known to enhance the immune response. Probiotic administration has been shown to increase the levels of various cytokines, such as IFN-γ and IL-6, which are crucial for mounting an effective immune response against infections (Yan and Polk, 2011; Satrio, *et al*., 2019). Probiotics can also stimulate the production of secretory IgA (sIgA), which plays a vital role in

mucosal immunity by preventing pathogen adhesion and invasion (Simon *et al*., 2011). This is particularly important in the context of Shigella, as sIgA antibodies can significantly reduce the severity of infection and inflammatory responses (Simon *et al*., 2011).

Moreover, Lactobacillus has been associated with improved macrophage function, enhancing their ability to scavenge free radicals and respond to bacterial infections (Trikha *et al*., 2017). This antioxidative capacity is essential for controlling inflammation and promoting recovery from infections. The modulation of immune responses by Lactobacillus is further supported by findings that show increased dendritic cell activity and enhanced adaptive immune responses following probiotic (Galdeano *et al*., 2011).

The potential of Lactobacillus as a therapeutic agent against Shigella infections is underscored by studies demonstrating its efficacy in both in vitro and in vivo models. For instance, Trikha et al. reported that Lactobacillus strains isolated from human infants could effectively remediate Shigella dysenteriae infections in macrophages, highlighting the probiotics' role in enhancing host defenses against intracellular pathogens (Trikha et al., 2017). Furthermore, the use of Lactobacillus in conjunction with other probiotics has been shown to provide a synergistic effect, enhancing overall pathogen inhibition (Piątek *et al*., 2019).

2.6 Immune Response

The immune response is a complex and dynamic process that involves both innate and adaptive immunity, which work together to protect the host from pathogens and maintain homeostasis. The innate immune system serves as the first line of defense, employing various pattern-recognition receptors (PRRs) to detect and respond to pathogens. Toll-like receptors (TLRs) are a critical component of this system, recognizing conserved structures in pathogens and initiating signaling pathways that lead to the activation of immune responses (Kawai and Akira, 2010).

Dysregulation of TLR signaling can result in inflammatory diseases or immunodeficiencies, highlighting the importance of maintaining immune homeostasis (Kawai and Akira, 2010).

In addition to TLRs, other factors such as glycosylation and the retromer complex have been shown to modulate innate immune responses. For instance, glycosylation plays a significant role in regulating immune signaling and maintaining homeostasis in Drosophila, a model organism for studying innate immunity (Yamamoto-Hino *et al*., 2015). The retromer complex has also been implicated in promoting immune quiescence by suppressing pathways that could lead to excessive immune activation (Zhou *et al*., 2013). This balance between activation and suppression is crucial, as hyper-activation of the immune response can lead to detrimental effects, such as tissue damage and chronic inflammation (Zhou *et al*., 2013).

The adaptive immune response, characterized by the activation of T and B lymphocytes, is also influenced by the innate immune system. For example, the presence of specific cytokines, such as interleukin-6 (IL-6), can shape the adaptive response during infections like leptospirosis, where both cellular and humoral immune responses are involved (Limothai *et al*., 2021). Furthermore, the interplay between different immune cells, such as neutrophils and regulatory T cells (Tregs), is essential for orchestrating an effective immune response. Neutrophils not only respond to infections but also play a role in shaping T cell responses by migrating to lymph nodes and influencing T cell polarization (Beauvillain *et al*., 2011; Wang and Arase, 2014). Furthermore, the adaptive immune system, or acquired immunity, constitutes a remarkable complex series of mechanisms, uniquely developed throughout the time that evolution required. In its broadest sense, we say that the adaptive immune system is a set of mechanisms used by vertebrates to identify and eliminate non-self-elements harmful to an individual's own body. Two other mechanisms make up the system that must be analyzed, even if briefly: non-specific

immunity or innate immune system; and tolerance phenomenon, or immune tolerance. Overall, both active immunity and passive immunity are, in turn, responsible for the immune memory of those who acquired the property of recognition of non-self-antigens. The most important components of the adaptive immune system are lymphocytes or cells that, through an incredibly large repertoire of antigen receptors, are responsible for recognizing and effectively eliminating a wide variety of antigens and associated with the lymphocytes.

In addition, lymphocytes are activated by antigen binding and proliferate in antigen-specific forms to combat a potentially damaging non-self-entity. Such activation can occur due to the recognition of necessity, skipping damaged cells or abnormal molecules or, less frequently, by autorecognition or autoimmune responses. Regarding the antigen-binding lymphocytes, it is possible to say that several possible forms occupied by receptors will be created by germline gene segments, and this can happen even before antigen exposure. This means that antigen recognition occurs in two different ways, with each one using a receptor in completely opposite configurations. That means that the antigen has to be localized in two specific areas. T receptors usually recognize small peptide fragments displayed by the proteins of infected cells, probably providing a mechanism of antiviral immunity unparalleled by other animals. This is also related to a very strange reaction of fishes to this specific factor. B cell receptors create innumerable forms capable of highly specific recognition of almost any chemical structure encoded in the genomic DNA, showing low relative affinity for most antigens.

In the context of cancer, the immune response can be both protective and detrimental. Tumors can exploit immune regulatory mechanisms to evade detection and destruction by the immune system. For instance, the expression of immune checkpoints such as PD-1 and CTLA-4 can inhibit T cell activity, leading to adaptive resistance against immunotherapy (Benci *et al*., 2016;

Koyama *et al*., 2016). Understanding the genetic determinants of immune phenotypes in tumors can provide insights into how to enhance the efficacy of immunotherapies by reactivating antitumor immune responses (Hendrickx *et al*., 2017; Charoentong *et al*., 2017).

Overall, the immune response is a finely tuned system that requires precise regulation to effectively combat pathogens while avoiding excessive inflammation or autoimmunity. Advances in our understanding of the molecular mechanisms underlying immune regulation continue to inform therapeutic strategies for infectious diseases and cancer.

2.6.1 T Cells

T cells are so named because they develop to maturity in the thymus. The thymus, an epithelial structure at the base of the throat, is large in infants and children, and shrinks with age until very little remains in old age. It is important for the development of tolerance, the characteristic held in common by both types of lymphocytes, and also contributes the majority of circulating T lymphocytes. The precursors of T cells develop in the bone marrow, like those of B cells, but in response to different environmental signals, leave the bone marrow and travel to the thymus, where they mature. T cells provide a whole host of protective functions, including helping activated B cells to make antibodies, destroying cells infected with viruses, bacteria, or other microorganisms, and preventing immune reactions against self-antigens, which would lead to diseases called autoimmunity's. Most T cells recognize small protein pieces, called peptides. The peptides are generated by the cells from proteins and are displayed on the surface of the cell. The peptides come from both the self-proteins normally produced by the cell's own ribosomes and from foreign proteins produced by intracellular viral, bacterial, and parasitic invaders. T cells bearing receptors that recognize self-peptides with too high an affinity is typically deleted during the course of their development within the thymus, a process called negative selection. However,

some of the self-peptide/MHC recognition is also necessary to establish or maintain lymphocyte populations capable of being active in the face of infection. Positive selective events may regulate the size and distribution of the mature T cell repertoire by directing T cells with a given affinity range to different maturation pathways, as well. T cells are critical for the development of an immune response. They protect us from infection by recognizing foreign antigens but ignoring self. Five fundamental properties of T cells are that they recognize antigens as short peptides, only when these peptides are complexed with class I or class II MHC molecules, that they bear clonotypic antigen receptors consisting of two distinct chains with fixed epitope binding specificities, which are generated by somatic gene rearrangement, that they are trained to be non-reactive to all self, and that they are trained to be reactive toward non-self. T cell clonotype ultimately means immune reactivity tailored specifically to non-self. Immune reactivity toward self, which is necessary for development and tissue maintenance, has to be prevented, and the immune system has evolved several mechanisms to achieve this. T cells use their specific T cell receptor (TCR), which they express as part of the complex with CD3, to recognize peptides. Thus, the antigens recognized are in the form of small peptides, which T cells see only in the form of a complex where the peptide is bound to a large, invariable receptor protein encoded by a family of genes known as the MHC. In contrast to secreted antibodies, TCRs do not recognize intact native antigens but need them to be degraded into small peptide fragments. They do that differently, using different structural principles and different classes of membrane proteins.

2.6.2 B Cells

The role of B cells in the immune system goes beyond the production of antibodies; they are just as important as their T cell counterparts. Indeed, the production of antibodies is just the tip of the iceberg, as B cells produce a wide range of molecules that can have both pro-inflammatory and anti-inflammatory effects. It is these other functions, and also the ability of some B cell subsets to regulate the immune response, that have led to widespread interest in exploring the possible therapies that could exploit the beneficial effects of B cells in treating human diseases. B cells get their name from the bursa, an organ in birds where B lymphocytes were first discovered. This discovery set back progress in understanding B lymphocytes in mice and humans, as these species do not have a bursa. In fact, anatomically similar structures in mammalian species have now been identified as the site of B lymphocyte development. As research progressed, it was discovered that the bursa in birds and the mammalian equivalent are the sites of B cell production, and where the antibody genes were being customized by gene conversion and somatic hypermutation. Unlike T lymphocytes, which could work for years, B lymphocytes and their products work for only a few days before they are removed from the bloodstream and renewed again from bone marrow hematopoietic stem cells. Furthermore, B cells is a crucial component of the adaptive immune system, undergo a complex differentiation process that is influenced by various cytokines and signaling pathways. The earliest stages of B cell development are critically dependent on interleukin-7 (IL-7), which is essential for the transition from common lymphoid progenitors to pro-B cells. Studies have shown that deficiencies in IL-7 or its receptor led to significant impairments in B cell development, resulting in the accumulation of immature B cells and a failure to produce functional B cells in peripheral tissues (Miller *et al*., 2002; Oliver *et al*., 2004). IL-7 not only promotes the survival of precursor B cells but also

works synergistically with other factors, such as Flt3 ligand, to facilitate their differentiation into more mature B cell stages (Oliver *et al*., 2004).

The differentiation of B cells is also modulated by various surface receptors and transcription factors. For instance, the B cell receptor (BCR) plays a pivotal role in mediating B cell maturation and activation. It has been demonstrated that signals from the BCR are crucial for the arrest of B cell differentiation, while the pre-B cell receptor does not have the same effect (Ceredig et al., 2000). Additionally, the transcription factor T-bet has been implicated in the differentiation of B cells during Th1-biased immune responses, indicating that the programming of B cells is closely linked to the type of T helper cell response being generated (Cooper *et al*., 2018).

Moreover, the role of cytokines such as IL-10 and IL-2 in B cell differentiation cannot be overlooked. IL-10 has been shown to induce differentiation of memory B cells into plasma cells, thereby influencing the antibody production profile (Choe & Choi, 1998). Conversely, IL-2 can directly affect B cell differentiation, particularly in the context of T cell interactions, highlighting the interconnectedness of T and B cell responses (Bich-Thuy & Fauci, 1985). This interplay is further exemplified in conditions such as systemic lupus erythematosus (SLE), where B cell responses are altered, leading to hyperactivity and impaired differentiation (Jandl *et al*., 1987).

Recent advancements in understanding the molecular mechanisms governing B cell differentiation have also highlighted the role of long noncoding RNAs (lncRNAs) and microRNAs. These regulatory molecules are involved in the fine-tuning of gene expression during B cell development, affecting the transition from naive B cells to memory or plasma cells (Pan *et al*., 2022; Brazao *et* al., 2016). The intricate regulatory networks that include these

noncoding RNAs underscore the complexity of B cell biology and the precision required for effective immune responses.

2.7 Gut Microbiota

The gut microbiota, a complex community of microorganisms residing in the gastrointestinal tract, plays a crucial role in host health and disease. Recent studies have highlighted the importance of gut microbiota diversity and composition in various biological contexts, including social relationships, diet, and disease susceptibility.

Research has shown that gut microbiota composition can be influenced by social relationships, particularly in humans. Dill-Mcfarland *et al*. (2019), found that close social ties, such as marriage, correlate with specific gut microbiota profiles, suggesting that social interactions may serve as a mediating pathway affecting health outcomes through microbiota changes (Dill-McFarland *et al*., 2019). This aligns with findings that highlight the role of gut microbiota in modulating immune responses and overall health, underscoring the need for further longitudinal studies to explore these connections in diverse populations (Dill-McFarland *et al*., 2019).

Diet is another significant factor affecting gut microbiota diversity. Studies indicate that a more varied diet is associated with greater microbial diversity, which is generally beneficial for host health (Li *et al*., 2016). For instance, Li et al. demonstrated that diet diversity influences the beta diversity of gut microbiota in pikas, suggesting that animals consuming a wider variety of foods harbor a more diverse microbial community (Li *et al*., 2016). This notion is supported by research on fish, where dietary habits were shown to correlate with microbial diversity, indicating that omnivorous diets promote a richer gut microbiota compared to carnivorous or herbivorous diets (Sun *et al*., 2021). Moreover, the implications of gut microbiota diversity

extend to various health conditions. Yang et al. reported a link between gut dysbiosis and hypertension, emphasizing that a decrease in beneficial bacteria such as Bifidobacterium can lead to adverse health outcomes (Yang *et al*., 2015). Similarly, reduced gut microbiota diversity has been associated with chronic diseases, including obesity and inflammatory bowel disease (IBD), highlighting the critical role of a balanced microbiome in maintaining health (Yoon *et al*., 2018). The relationship between gut microbiota and metabolic diseases is further supported by findings that suggest low microbial diversity correlates with increased prevalence of such conditions (Badal *et al*., 2020).

In addition to diet and social factors, the development of gut microbiota is influenced by early life experiences, including delivery mode and weaning. Infants born via cesarean section often exhibit lower gut microbiota diversity compared to those delivered vaginally, which can have long-term health implications (Lee et al., 2016). Furthermore, the transition from breast milk to solid food during weaning is characterized by significant changes in gut microbiota composition, reflecting a maturation process that is crucial for immune system development (Hasebe *et al*., 2022).

2.7 The Gut Microbiome

The gut microbiome, a complex community of microorganisms residing in the gastrointestinal tract, plays a crucial role in various physiological processes, including metabolism, immunity, and mental health. The composition and diversity of the gut microbiome can significantly influence these functions, highlighting its importance in maintaining overall health.

The gut microbiome is initially established through vertical transmission from the mother, with factors such as prenatal stress potentially altering its development. Studies have shown that fetuses exposed to maternal stress exhibit a gut microbiota with reduced levels of beneficial

bacteria like Bifidobacterium, which is essential for gut health and immune function (Clapp *et al*., 2017). This early establishment is critical, as the gut microbiome continues to evolve throughout an individual's life, influenced by diet, environment, and lifestyle choices.

The diversity of the gut microbiome is a key indicator of its health. Higher alpha diversity, which refers to the variety of species present, is often associated with better health outcomes. For example, a study found that individuals with greater gut microbiome diversity exhibited more favorable responses to immunotherapy in cancer treatment, suggesting a link between microbial diversity and immune function (Shoji *et al*., 2023). Conversely, reduced diversity has been linked to various health issues, including metabolic disorders and mental health conditions (Peirce and Alviña, 2019; Weersma *et al*., 2020). This complexity underscores the need for a nuanced understanding of how specific microbial taxa contribute to health and disease. Inaddition, the gut microbiome's influence extends to mental health, with emerging evidence supporting the gut-brain axis hypothesis. Alterations in gut microbiota composition have been associated with mental health disorders such as anxiety and depression (Lee *et al*., 2020; Pedroso *et al*., 2022). For instance, individuals with a Prevotella-dominant gut microbiome have been linked to improved emotional well-being, indicating that specific microbial profiles may promote mental health (Lee *et al*., 2020). Additionally, dietary interventions, such as increased intake of unpasteurized milk, have been shown to enhance beneficial bacteria like *Lactobacillus* species, potentially improving gut health and, by extension, mental health outcomes (Butler *et al*., 2020).

The relationship between gut microbiome composition and physical health is also significant. Recent studies have identified specific gut microbes associated with muscle strength, suggesting that the gut microbiome may influence physical performance (Ahn et al., 2023; Ahn, 2024). This

connection highlights the potential for microbiome-targeted interventions to enhance physical health, particularly in populations at risk for muscle weakness

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study Area

The laboratory analysis for this study was conducted at the Microbiology Laboratory, University of Benin, and the Animal House and Laboratory of the Faculty of Pharmacy, University of Benin, Edo State, Nigeria. Ethical approval was obtained for the use of laboratory and animals. These facilities provided the controlled environments and equipment's necessary for microbial, pathological, and immunological investigations.

3.2 Sample Collection and Preparation

Lactobacillus casei and *Shigella flexneri* were obtained from Medical and Parasitology Laboratory at Bayelsa State and were transported to the laboratory under aseptic conditions and cultured on de Man, Rogosa, and Sharpe (MRS) Agar and *Salmonella Shigella* Agar for confirmation of the presumptive isolates. The isolate was sub-cultured on nutrient agar and maintained for subsequent experimental use.

3.3 Experimental Design

The experimental design was structured to evaluate the immunomodulatory and therapeutic effects of *Lactobacillus casei* in mitigating *Shigella flexneri* infections in Wistar albino rats. The study involved five groups, each comprising four rats:

1. Control Group: Received no treatment or infection, serving as the baseline.

2. Challenged Group: Exposed to *Shigella flexneri* infection without any treatment from day 7 - 14 days

3. Probiotic Group: Challenged with *Lactobacillus casei* for seven days

4. Prophylactic Group: Pre-challenged with *Lactobacillus casei*. for seven (7) days before being administered *Shigella flexneri* to assess preventive effects.

5. Antibiotic Group: Challenged with *Shigella flexneri* and treated with a standard antibiotic (ciprofloxacin) to establish a therapeutic comparison.

Ethical approval was obtained from the institutional animal care and use committee (IACUC), and all procedure were conducted in compliance with institutional ethical standard. All rats were housed in grouped cages under controlled environmental conditions, including a 12hours light/dark cycle and access to food and water. Rats were bought from Pharmacy Animal House and kept in the laboratory for 7days to acclimatize.

3.3.1 Selection and Housing of Rats

Forty healthy Wistar albino rats, of eight weeks old and weighing between 150-200 grams, were selected for uniformity in physiological parameters. Animals were housed in polypropylene cages with sterilized bedding to reduce contamination risk. Rats were fed with a standard rodent chow diet formulated to meet their nutritional needs and were provided with filtered water. All animals were acclimatized to laboratory conditions for 14 days before starting experiments to mitigate stress-induced variability.

3.3.2 Preparation of suspension

Following acclimatization, rats in the challenged group and prophylactic group were challenged with *Shigella flexneri* by intraoral injection. The infection model aimed to simulate systemic infection. A single colony of *Shigella flexneri*. Was inoculated in 10ml sterile saline water and shake and serial dilution was carried out. The *Shigella flexneri* suspension (1×10^8 CFU/ml) was carefully administered, and signs of infection were monitored over the next 24-48 hours. Signs

such as weight loss, lethargy, and fever were recorded, providing baseline data for subsequent analysis of the treatment effects.

3.4 Administration of *Lactobacillus casei*

The *Lactobacillus casei* preparation was suspended in sterile phosphate-buffered saline (PBS) to achieve a concentration of 1×10^8 CFU/ml. The probiotic Group was challenged with *Lactobacillus casei* for seven days. The prophylactic group was pre-challenged with *Lactobacillus casei* for seven (7) days before being challenged with *Shigella flexneri* to assess preventive effects. This pretreatment was intended to promote gut colonization and prime the immune response against potential pathogens.

3.5 Microbial Analysis

3.5.1 Preparation and Sterilization of Materials

All glassware and instruments used during the study were sterilized in an autoclave at 121°C for 15 minutes. Sterilized disposable materials such as syringes, needles, and gloves were used to ensure aseptic handling. Media used in the study, including MRS agar, Nutrient Agar, and Salmonella Shigella Agar, were prepared according to the manufacturer's instructions and autoclaved at 121°C for 15 minutes before use.

3.5.2 Microbial Analysis of Stool Samples

Stool samples were collected from rats prior to induction with *Shigella flexneri* and Lactobacillus spp., after induction with *Shigella flexneri*, and antibiotic treatment. Approximately 1 g of stool was homogenized in 9 mL of sterile PBS using a vortex mixer to ensure thorough dispersion. A tenfold serial dilution was performed by transferring 1 mL of the stool homogenate into 9 mL of sterile PBS, creating dilutions from 10^1 to 10^6. From the 1 dilution, 0.1 mL was plated onto

Nutrient agar plates and incubated at 37°C for 24hrs. Colonies were picked from the nutrient agar and inoculated on salmonella shigella agar and incubated for24hrs and colonies were picked and counted

$$\frac{cfu}{g} = \frac{number\ of\ colonies\ x\ dilution\ fold/series}{volume\ of\ inoculum}$$

3.6. IDENTIFICATION OF BACTERIA

3.6.1 GRAM STAINING

Thin smears of the bacterial isolates were made from the pure culture on clean, grease free slide. The smears were air dried and then heat fixed by passing the slide over flame. The smears were flooded with crystal violet for 60 seconds, drained and flooded with gram's iodine for another 60 seconds then rinsed with distilled water. The smears are then decolorized using 70% alcohol for 5 seconds and quickly rinsed with distilled water. The smears were then counter stained with safranin for 30 seconds, rinsed with distilled water and allow to air dried. The slides were examined under the oil immersion(X100) objective. The gram-positive cells appear blue while the gram-negative cells were indicated with a pink coloration.

3.6.2. Potassium Hydroxide (KOH) test

Two drops of 3% solution of KOH were applied on a clean glass slide and a loopful of pure bacterial growth was stirred in a circular motion in the slide. The loop was occasionally raised and observed for the presence of a string of the mixture. The solution was observed to be of a viscous and mucoid consistency indicating a Gram-negative bacterium. No reaction (absence of stringing) indicates a Gram-positive bacterium (Roberts and Sandle, 2008).

3.6.3. BIOCHEMICAL TEST

Biochemical characteristics of each isolate carried out to identify them include catalase test, citrate utilization test, oxidase test, urease test, methyl red test, indole test, coagulase test, sugar fermentation, starch hydrolysis and Triple sugar iron fermentation.

3.6.3.1 CATALASE TEST

Catalase test was carried out by making a suspension of fresh culture of the test organisms using sterile distilled water on a clean glass microscope slide and few drops of hydrogen peroxide (H_2O_2) were added using a dropping pipette. Formation of bubbles indicates positive result. Lack of bubbles indicates negative result.

3.6.3.2 CITRATE UTILIZATION TEST

The citrate utilization test is a part of the test used to differentiate organisms on their ability to utilize citrate as the primary energy source. A citrate test was performed to differentiate members of Enterobacteriaceae capable of fermenting citrate in the presence of the enzyme citrate.

Simon's citrate agar contained citrate as significant energy and was prepared for inoculation on Petri dishes. Well-prepared and sterilized citrate agar plates were inoculated from the pure isolated culture by streaking the surface with a sterilized loop. The plates were then incubated at 37°C for 24 hours. There were changes in colour due to bacterial growth of the organisms on the medium due to citrate metabolism, which gave a positive citrate test. The shift in pH turns the bromothymol blue indicator in the medium from green to blue (positive result). A negative test was demonstrated with no growth, no colour change, or the colour of the medium remains green.

3.6.3.3. OXIDASE TEST

The oxidase test was carried out to detect the presence of a cytochrome oxidase or indophenol oxidase that will catalyze electrons between electron donors in the bacteria and a redox dye known as tetramethyl-p-phenylene-diamine. The dye would be reduced to deep purple colour if yielded to positive reactions.

Several reagents can be used for this study but Kovacs oxidase reagent: 1% tetra-methyl-p-phenylenediamine dihydrochloride in water, was used. The filter paper was saturated with a Kovacs oxidase reagent solution, and a speck of the pure culture was smeared on it with a platinum loop. It was allowed and observed for colour development within 10 - 60 seconds. The appearance of a deep purple-blue/blue colour indicated oxidase production and the negative result was when no colour changed (Fawole and Oso, 2007).

3.6.3.4. INDOLE TEST

Spot indole test was carried out using a fresh culture of the test organism. Several drops of 1% p-dimethy laminocinnamaldehyde reagent were placed on a piece of filter paper. A loopful culture of the test organism was rubbed on the reagent saturated area of the filter paper. Positive result is shown by the presence of a blue to blue-green colour change within 2-3 minutes while negative results remain colourless or appears light pink

3.6.3.5. Triple sugar iron (TSI) agar test

An agar slant prepared of a SI agar was used in carrying out this test in a sterile test tube at a slanted angle. The slanted medium was inoculated with TSA pure culture using a straight inoculation needle by stabbing first through the center to the bottom of the tube and streaking the agar slant's surface. After inoculations, the test tubes were covered with foil paper and left at an ambient temperature of 36°C to incubate for 24 hours. Reactions on test tubes were examined

and sugar fermentations were indicated by the production of HS, gas and a change in colours from red (alkaline) to yellow (acid). When an alkaline/acid (red top/yellow bottom) slant reaction appeared, it only indicated dextrose (glucose) fermentation. When an acid/acid (yellow top/yellow bottom) slant reaction appeared, it showed the fermentation of dextrose, lactose and/or sucrose. The appearance of an alkaline/alkaline (red top/red bottom) slant reaction represented the absence of sugar fermentation. The blackening of the medium in the slant indicated H2S production. Bubbles, cracks, or bottom-raised space in the slanted agar indicated gas production (formation of CO_2 and H_2).

3.7 Histopathological examination

Segments of colon, and rectum were surgically removed, washed, and then fixed in 4% buffered formalin and were inspected for signs of inflammation and hemorrhage. Sections were stained with hematoxylin and eosin, and examined under a microscope for pathological changes. The segments were inspected for signs of mucosal edema, exudation, hemorrhage, ulceration, necrosis, and perforation. The segments were cut using a microtome and mounted onto clean, grease-free glass slides. The tissue sections were stained with hematoxylin and eosin (H and E) to highlight cellular and structural features for microscopic examination. Each slide was analyzed under a light microscope by a qualified pathologist who was blinded to the treatment groups to ensure unbiased evaluation.

The histopathological parameters assessed included evidence of cellular infiltration (inflammatory response), necrosis (cell death, and architectural disruption (alteration of tissue structure). A standardized scoring system was employed to quantify tissue damage, with scores ranging from 0 to 4:

0: No observable damage

1: Mild damage with slight inflammatory changes

2: Moderate damage with noticeable cellular infiltration and minor structural alteration

3: Significant tissue damage, marked necrosis, and disrupted architecture (Rabbani, *et al*., 1995)

4: Severe tissue destruction with extensive necrosis and loss of structural integrity

Findings were systematically documented for each tissue type and compared across experimental groups to evaluate the protective effects of *Lactobacillus casei*.

CHAPTER FOUR

RESULTS

Table 4.1 shows the Cultural, Morphological and Biochemical Characteristics of *Lactobacillus casei* and *Shigella flexneri*

Shigella flexneri and *Lactobacillus casei* were identified using standard cultural, morphology and biochemical test. Various differential media (Man–Rogosa–Sharpe agar (MRS) and Salmonella Shigella Agar) were also used for identification.

The physical assessment of albino rats before inducing with *Shigella flexneri* and *Lactobacillus casei* at day zero of the experiment is represented on Table 4.2. which shows the physical parameter of Wistar albino rats in each group. The temperature 36.0 ^{O} C and the rats stool observed was normal in the control group, challenged group, probiotic group, prophylactic group and antibiotic group. The observed weight was highest in the challenged group (239.3g) and probiotic group (239.9g) while the control group had a weight of 180.3g.

The physical assessment of Wistar albino rats after inducing with *Shigella flexneri* (challenged group and antibiotic group) and *Lactobacillus casei* (probiotic group and prophylactic group) at day seven of the experiment is represented on Table 4.3. It was observed that there was no temperature change in the control group (36.0 ^{O} C) but there was an increase in temperature in the challenged group (37.2 ^{O} C), probiotic group (37.3 ^{O} C), prophylactic (36.8 ^{O} C) and antibiotic group (38 ^{O} C). A decrease in the weight of the Wistar albino rats in challenged group (148.4g), prophylactic (141.3g) and antibiotic group (161.6g) was observed but the control group increased in weight (263.1g). The stool of the challenged group was slightly red and slightly light in the antibiotic group while the stool of control group and probiotic group were still normal as seen in day zero of the experiment.

The physical assessment of albino rats before inducing with *Shigella flexneri* (Prophylactic group) and antibiotic (antibiotic group) at day fourteen of the experiment is represented in Table 4.4. It was observed that the temperature (36.0 ^{O} C) and stool in the control group were the same and with a slight increase in the weight of the rats (265g) while there was an increase in the temperature in the challenged group (37.8 ^{O} C) and a decrease in the rat weight (142.3g). There was a decrease in temperature in the antibiotic group (37 ^{O} C) and increase in the rat weight (180g) and slight temperature increase the prophylactic group (37 ^{O} C) and increase in the weight (178g).

Physical assessment on albino rats after inducing with *Shigella flexneri* (D) and antibiotic (E) at day twenty-one is represented in Table 4.5. It was observed that the temperature (36.0 ^{O} C) and stool in the control group were the same with a slight decrease in the weight of the rats (260g) while there was a constant temperature in the challenged group (37.8 ^{O} C) and a decrease in the rat weight (140g). There was a constant in temperature in the antibiotic group (37 ^{O} C) and increase in the rat weight (182g) and slight temperature increase the prophylactic group (37 ^{O} C) and increase in the weight (180g). The antibiotic-treated group maintained a stable temperature (37.0°C), slightly above normal, which may indicate a lingering but controlled infection or an immune response. The weight of the rats increased (182g), suggesting that antibiotic treatment helped mitigate the infection, allowing for improved food intake and overall health recovery.

Colony pathology of Wistar albino rat is represented in Figure 4.6. which shows the hematoxylin-and-eosin-stained colonic section of Wistar albino rat challenged with *Shigella flexneri* and *Lactobacillus casei.* The left hand shows the colon challenged with *Lactobacillus casei*. The colon appears normal and had no edema or disruption of the surface layer of epithelial cells and no ulcerations and cellular infiltrations in the lamina propria while the right hand shows

the colon challenged with *Shigella flexneri*. The colon appears unhealthy, light blue arrow indicated area of edema and deep ulcerations, cellular infiltrations in the lamina propria while the spleen in the control rats appears histologically normal. The control spleen demonstrated a balanced healthy immune tissue were as the colon shows inflammation.

Mean heterotrophic bacteria count of Wistar albino rat stool is represented in Table 4.6. which shows the bacteria load of Wistar albino rat stools before they were challenged with *Shigella flexneri* and *Lactobacillus casei*. The mean heterotrophic bacteria count ranged between 10.6×10^7 - 12.9×10^7 while the mean of heterotrophic bacteria count of Wistar albino rat organ is represented on Table 4.7 which shows the bacteria load of the colon and liver challenged with *Shigella flexneri* and *Lactobacillus casei*. The mean heterotrophic bacteria load ranged between 47×10^3 cfu/g – 78.6×10^3 cfu/g and 39×10^3 cfu/g – 82×10^3 cfu/g respectively. The highest count was observed in the challenged group and the least count in the control group.

Table 4.1: Cultural, Morphological and Biochemical Characteristics of *Lactobacillus casei* and *Shigella flexneri*

Cultural characteristics		
Colour	Cream	Cream
Shape	Rhizoid	Circular
Elevation	Flat	Raise
Margin	Filamentous	Entire
Size	Small	Large
Morphological characteristics		
KOH	-ve	-ve
Gram stain	+ve	+ve
Cell morphology	Rod	Rod
Cellarrangement	Chain	Dispersed
Biochemical characteristics		
Catalase	+	+
Indole	-	+
Oxidase	+	-
Voges-proskauer	Variable	Variable
Spore forming	-	-
Glucose	+	+
Lactose	+	-
Sucrose	-	+
Mannitol	+	-
H2S production	-	-
Identity	*Lactobacillus casei*	*Shigella flexneri*

Table 4.2: Physical assessment on albino rats before inducing with *Shigella flexneri* and *Lactobacillus casei* at day zero

Group	**Temperature (°C)**	**Frequency of stool**	**Weight (g)**
A	36.0 ± 0.00	Normal	180.3
B	36.0 ± 0.00	Normal	239.3
C	36.0 ± 0.00	Normal	239.9
D	36.0 ± 0.00	Normal	232.8
E	36.0 ± 0.00	Normal	250

KEY:

A = Control group

B = Challenged group

C = Probiotic group

D = Prophylactic group

E = Antibiotic group

Table 4.3: Physical assessment on albino rats after inducing with *Shigella flexneri* (B and E) and *Lactobacillus casei* (C and D) at day seven

Group	**Temperature (°C)**	**Frequency of stool**	**Weight (g)**
A	36.0 ± 0.00	Normal	263.1
B	37.2 ± 0.66	Slightly red	148.4
C	37.3 ± 0.75	Normal	229.7
D	36.8 ± 0.54	Normal	141.3
E	38 ± 0.75	Slightly light	161.6

KEY:

A = Control group

B = Challenged group

C = Probiotic group

D = Prophylactic group

E = Antibiotic group

Table 4.4: Physical assessment on albino rats before inducing with *Shigella flexneri* (D) and antibiotic (E) at day fourteen

Group	**Temperature (°C)**	**Frequency of stool**	**Weight (g)**
A	36.0 ± 0.00	Normal	265
B	37.8 ± 0.66	Slightly red	142.3
C	37.3 ± 0.75	Normal	235
D	37 ± 0.54	Normal	178
E	37 ± 0.75	Normal	180

KEY:

A = Control group

B = Challenged group

C = Probiotic group

D = Prophylactic group

E = Antibiotic group

Table 4.5: Physical assessment on albino rats after inducing with *Shigella flexneri* (D) and antibiotic (E) at day twenty-one

Group	**Temperature (°C)**	**Frequency of stool**	**Weight (g)**
A	36.0 ± 0.00	Normal	260
B	37.8 ± 0.66	Slightly red	140
C	37.3 ± 0.75	Normal	236
D	37 ± 0.54	Normal	180
E	37 ± 0.75	Normal	182

KEY:

A = Control group

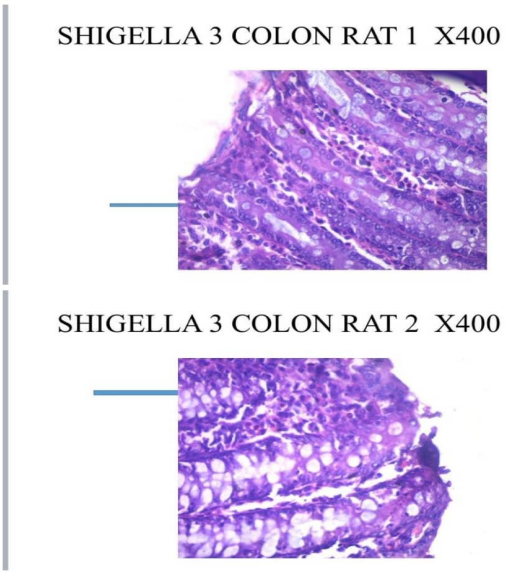
B = Challenged group

C = Probiotic group

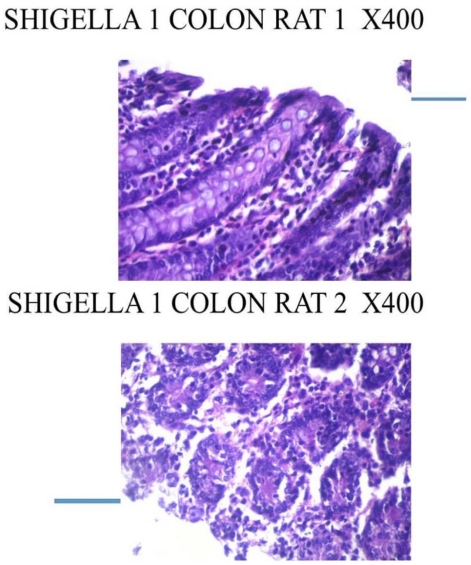
D = Prophylactic group

E = Antibiotic group

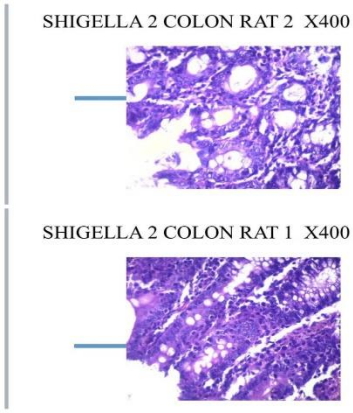
Figure 4.6: Colonic pathology of Wistar albino rats in challenged group, probiotic group and control group



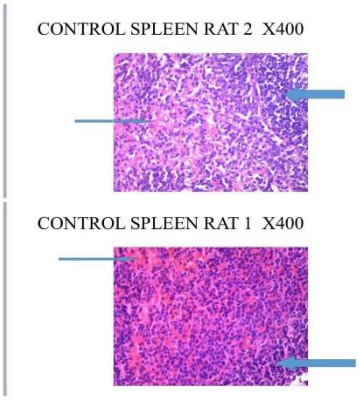
Challenged group



Antibiotic group

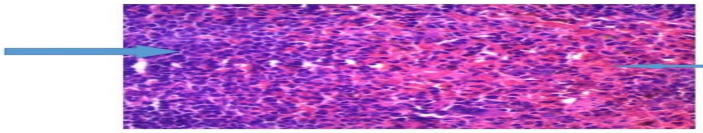


Prophylactic group

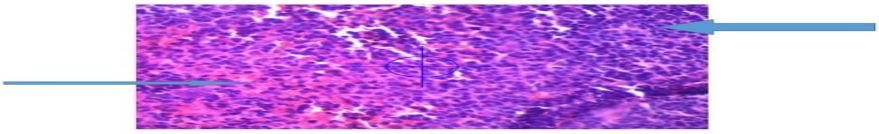


Control group

LACTO B SPLEEN RAT 1 X400

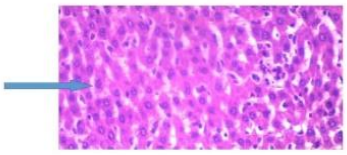


LACTO B SPLEEN RAT 2 X400



Probiotic group

SHIGELLA 1 LIVER RAT 2 X400



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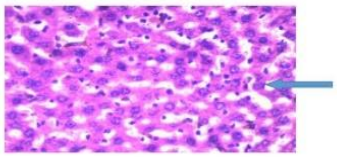
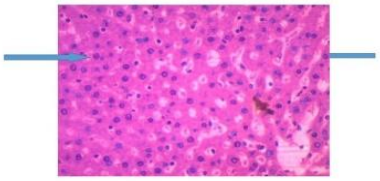


Antibiotic group

SHIGELLA 2 LIVER RAT 2 X400



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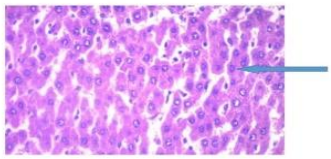
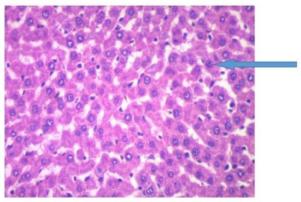


Prophylactic group

SHIGELLA 3 LIVER RAT 1 X400



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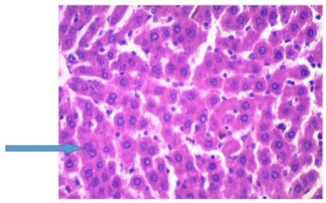
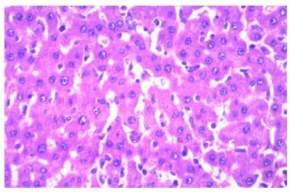
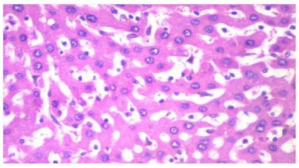


Challenged group

LACTO B LIVER RAT 2 X400

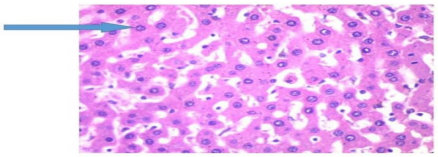


LACTO B LIVER RAT 1 X400

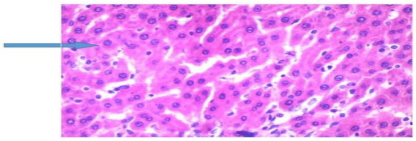


Probiotic group

LIVER CONTROL RAT 1 X400



LIVER CONTROL RAT 2 X400



Control group

Table 4.6: Mean heterotrophic bacteria count of Wistar albino rat stool

Rats stool samples	Day zero	Day seven	Day fourteen	Day twenty-one
Control group	11.9×10^{7}	11.2×10^{7}	8.7×10^{7}	$9. \times 10^{7}$
Challenged group	14.4×10^{7}	11.5×10^{7}	7.3×10^{7}	6.9×10^{7}
Probiotic group	13.9×10^{7}	12.0×10^{7}	6.8×10^{7}	6.5×10^{7}
Prophylactic group	15.9×10^{7}	13.9×10^{7}	8.7×10^{7}	8.5×10^{7}
Antibiotic group	16.6×10^{7}	15.5×10^{7}	6.7×10^{7}	7.0×10^{7}

Table 4.7: Mean heterotrophic bacteria count of Wistar albino rat organ

Group	Colon ($\times 10^3$) cfu/g	Liver ($\times 10^3$) cfu/g
Control group	47 ± 0.007	39 ± 0.035
Challenged group	78.6 ± 0.028	82 ± 0.042
Probiotic group	51 ± 0.014	51 ± 0.042
Prophylactic group	71 ± 0.049	57 ± 0.021
Antibiotic group	68 ± 0.028	58 ± 0.01

CHAPTER FIVE

DISCUSSION

The role of *Lactobacillus* species in enhancing immunity against Shigella infection in rats has drawn significant attention in recent research. *Lactobacillus* species, a probiotic, have been shown to exert protective effects against various pathogens, including *Shigella*, through multiple mechanisms that enhance the host's immune response. This study focused on investigating the potential of *Lactobacillus casei* as probiotics to enhance immune responses in Wistar albino rats against *Shigella flexneri* infection. The result presented in Table 4.1, shows the cultural, morphological and biochemical characteristics of *Lactobacillus casei* and *Shigella flexneri* that were purchased. Standard cultural, morphological, and biochemical tests, along with differential media (Man–Rogosa–Sharpe agar (MRS) and Salmonella Shigella Agar) were used for confirmation of the identification. Al-Muhtaseb *et al.* (2019) and Sokol *et al.* (2017) reported similar cultural characteristics for *Lactobacillus casei* on MRS agar, where it typically forms colonies that are creamy or white, with a smooth and slightly raised appearance.

The result presented in Table 4.2, shows the physical parameter of Wistar albino rats in each group. There was no mortality and nasal bleeding in all the group, but there was hair loss and slight red in the feces colour in Wistar albino rats challenged with *Shigella flexneri*. This is in accordance with the finding of Pradhan *et al*. (2016), who reported a hair loss in mice infected with *Shigella flexneri*. Furthermore, this could potentially be attributed to stress-induced alopecia. Stress caused by infection can lead to hormonal changes, especially an increase in cortisol, which might cause hair follicles to enter the resting phase and eventually shed hair. Kumaresan *et al.* (2014), also reported the physical parameters of Wistar albino rats, were closely monitored after infection with various pathogens. Hair loss observed could also be a result of the stress response

activated by *shigella* infection. The immune system's activation during an infection can trigger various physiological changes, including changes to the skin and hair. This finding is also similar to the work of Meyers *et al.* (2020), who reported on the effects of *E. coli* infection in rats and noted that stress caused by bacterial infection could result in hair loss, particularly in cases where the animal's immune system is actively responding to the pathogen. This response may also involve an increase in inflammatory cytokines, which can affect hair follicle cycling and cause hair shedding. While no mortality and nasal bleeding was observed, signs like reduced activity, and weakness were observed in the rats challenged with *shigella flexneri*.

The result presented in Table 4.3, show the colonic contents of Wistar albino rats challenged with *Shigella flexneri* and *Lactobacillus casei*. Trace of blood and heavy number of macrophages was observed in the colon of Wistar albino rats challenged with *Shigella flexneri* and not observed in Wistar albino rats challenged with *Lactobacillus casei*. The present of trace of blood and heavy number of macrophages in the colon of Wistar albino rats challenged with *Shigella flexneri*, indicated that there were immune responses in the rats. This aligns with the research of Steinhauer *et al*., (2022), suggesting that *Shigella flexneri* is a pathogenic bacterium capable of inducing significant inflammation and immune response. *Shigella* infections have been shown to induce a strong local inflammatory response, including the infiltration of macrophages and other immune cells (Steinhauer *et al*., 2022). These findings confirm that *Shigella flexneri* can damage the colon, triggering an inflammatory response, with the presence of blood and macrophages noted in the current study. Conversely, the absence of blood and macrophage infiltration in rats challenged with *Lactobacillus casei* reflects its potential protective effects. *Lactobacillus casei*, a probiotic strain, has been widely studied for its ability to modulate immune responses and maintain gut health. Previous studies have highlighted that *Lactobacillus casei*. can enhance

intestinal barrier function, reduce inflammation, and prevent infections by balancing the gut microbiota (Jiang *et al*., 2021). Which is similar to the study by Zhang *et al*. (2020), reported that *Lactobacillus casei* significantly reduced inflammatory markers in animal models, which is in consonance with the findings in this present study where no immune responses were observed in the probiotic group. In contrast to the immune response seen in *Shigella flexneri*-infected rats, the control group, which was not exposed to either pathogen or probiotic, also showed no immune responses. This supports the notion that the absence of *Shigella flexneri* or *Lactobacillus casei* in the animals does not trigger any inflammatory or immune activity, indicating that immune responses are pathogen- or treatment-dependent.

The result presented in Figure 4.4, shows the hematoxylin-and-eosin-stained colonic section of Wistar albino rat challenged with *Shigella flexneri* and *Lactobacillus casei.* on the light hand, shows the colon of Wistar albino rat challenged with *Lactobacillus casei*. The colon appears normal and had no edema or disruption of the surface layer of epithelial cells and no ulcerations and cellular infiltrations in the lamina propria. While on the right hand, shows the colon challenged with *Shigella flexneri*. The colon appears unhealthy, Light blue arrow indicated area of edema and deep ulcerations, cellular infiltrations in the lamina propria. The presence of edema and cellular infiltration in the lamina propria of the *Shigella flexneri*-challenged rats is a hallmark of the inflammatory response triggered by this pathogen. Infected tissues often exhibit these signs due to the activation of the immune system, including the recruitment of neutrophils and macrophages, which attempt to contain the infection but also contribute to tissue damage (Ibarra *et al*., 2021). On the left hand, the colon of rats challenged with *Lactobacillus casei* appears to be unaffected by inflammation, supporting the potential anti-inflammatory and protective effects *Lactobacillus casei* as a probiotic. *Lactobacillus casei* has been shown to maintain the integrity

of the epithelial barrier, reduce inflammatory markers, and promote gut health (Jiang *et al*., 2021).

The lack of edema, ulcerations, and cellular infiltrations observed in this group suggested that *Lactobacillus casei* helps prevent the immune system from reacting excessively to stimuli, potentially offering a protective role against intestinal infections and inflammation (Zhang *et al*., 2020). The inflammatory response typically involves recruitment of immune cells to the site of infection, which can lead to tissue damage and cellular infiltrations in the lamina propria observed in the rats challenged with *Shigella flexneri*. In contrast, *Lactobacillus casei* could attenuate inflammation and protect the intestinal mucosa in animal models. Lactobacillus was associated with improved clinical outcomes in infected rats. *Lactobacillus casei* is linked to normalizing the colon function and reducing inflammatory markers in pathogen-challenged rats, which suggest a systemic effect on health and immunity (Amengialue *et al*., 2023). The presence of Lactobacillus not only aids in the direct inhibition of pathogens but also contributes to the overall resilience of the rats immune system against infections (Pan, 2022). The absence of edema, ulcerations, and cellular infiltrations in *Lactobacillus casei* group mirrors these findings, supporting the idea that *Lactobacillus casei* may enhance gut barrier function and prevent the inflammatory damage typically induced by pathogens like *Shigella*. Further studies have explored the role of *Lactobacillus casei*, in maintaining intestinal integrity and mitigating the effects of pathogen-induced inflammation (Zhang *et al*. 2020).

Physical assessment on albino rats after inducing with *Shigella flexneri* (D) and antibiotic (E) at day twenty-one is represented in Table 4.5. It was observed that the temperature (36.0 ^{O} C) and stool in the control group were the same with a slight decrease in the weight of the rats (260g) while there was a constant temperature in the challenged group (37.8 ^{O} C) and a decrease in the

rat weight (140g). There was a constant in temperature in the antibiotic group (37 ^{O} C) and increase in the rat weight (182g) and slight temperature increase the prophylactic group (37 ^{O} C) and increase in the weight (180g).

The result presented in Table 4.6, shows the bacteria load of Wistar albino rat stools before they were challenged with *Shigella flexneri* and *Lactobacillus casei* and during challenged period and after challenged. The mean heterotrophic bacteria count ranged between 7.3×10^{7} - 16.6×10^{7} reflects the bacterial populations in the rat stools at different stages of the experiment.

The increase in bacterial load during the challenge period is expected, particularly when considering the pathogenic effect of *Shigella flexneri*. *Shigella* is a facultative intracellular pathogen that can proliferate in the intestinal tract, potentially leading to an alteration in the gut microbiome. The high bacterial count during infection could be attributed to both the proliferation of *Shigella flexneri* and the body's immune response, which may lead to changes in the bacterial communities as the immune system reacts to the pathogen. *Shigella* infections typically lead to increased bacterial proliferation in infected tissues due to the bacterium's ability to invade intestinal cells and resist host immune defenses. The elevated bacterial load in Cage 3 suggests a higher level of *Shigella* proliferation within the tissues. The higher bacterial counts could be indicative of an ongoing infection and the inability of the host's immune system to effectively limit bacterial growth during the challenge period.

In contrast, the administration of *Lactobacillus casei* likely has a different impact on the bacterial load. *Lactobacillus casei*, a well-known probiotic, has been shown to modulate the gut microbiota by enhancing the growth of beneficial bacteria and suppressing pathogenic bacteria (Jiang *et al*., 2021). A more stable or balanced bacterial load observed during the *Lactobacillus casei* challenge period would be expected, as the probiotic helps maintain the diversity and

health of the intestinal microbiota. Furthermore, the lower bacterial count observed in the *Lactobacillus casei*-challenged group (Cage 4) aligns with the known role of probiotics in maintaining gut health. Probiotics such as *Lactobacillus casei* have been shown to exert antimicrobial properties and can help regulate the growth of pathogenic bacteria while promoting a balanced microbiota (Jiang *et al*., 2021). The lower bacterial load in Cage 4 may reflect the probiotic's role in controlling the growth of pathogenic bacteria and maintaining the homeostasis of the intestinal microbiota during the challenge period.

The administration of probiotics like *Lactobacillus casei* has been shown to help reduce pathogenic bacterial load. Jiang *et al*. (2021) repotred that *Lactobacillus casei* not only modulates the immune response but also supports the growth of beneficial bacteria while suppressing pathogens. The lower bacterial load in Cage 4 is in consonance with these findings, suggesting that *Lactobacillus casei* may have exerted a protective effect by keeping *Shigella flexneri* under control and potentially preventing its overgrowth in the rats' colon.

CONCLUSION

Lactobacillus casei demonstrates significant potential as a therapeutic agent to enhance immune responses, reduce inflammation, and protect against intestinal damage caused by *Shigella flexneri*. This study supports the growing body of evidence suggesting that probiotics can play a crucial role in modulating immune function and maintaining gut health in the face of pathogenic challenges. Further research is needed to explore the long-term effects and clinical applicability of *Lactobacillus casei* in managing enteric infections.

RECOMMEDATION

I recommend further research should be conducted to explore the long-term effects of Lactobacillus casei supplementation on immune function and gut microbiota and the potential use of Lactobacillus casei as a probiotic supplement for managing enteric infections should be investigated in clinical settings.

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