Evaluation of Probiotic Supplementation as an Intervention for Antibiotic-induced Stress in Murine Model

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May, 2023

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A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science inApplied Biosciences.

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CERTIFICATE OF ACCEPTANCE

Certified that final contents and form of BS thesis entitled **Evaluation of Probiotic Supplementation as an Intervention for Antibiotic-induced Stress in the Murine Model"** written by Ms Esha Maryam (Registration No 00000323649), Ms. Shanza Jabeen (Registration No. 00000324793), and Ms. Momna Nasir (Registration No. 00000322753) of ASAB, has been vetted by undersigned, found complete in all respects as per NUST Regulations, is free of plagiarism, errors and mistakes and is accepted as partial fulfillment for the award of BS degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis.

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DECLARATION

We, Esha Maryam, Shanza Jabeen, and Momna Nasir declare that all work presented in this thesis is the result of our own work. Where information has been derived from other sources, we confirm that this has been mentioned in the thesis. The work herein was carried out while we were undergraduate students at Atta-ur-Rahman School of Applied Biosciences, NUST under the supervision of Dr. Saadia Andleeb.

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Shanza Jabeen

Momna Nasir

DEDICATION

Dedicated to my exceptional parents and adored siblings whose tremendous support and cooperation led me to this wonderful accomplishment.

Acknowledgments

All Praise and Glory to Allah the Almighty, who blessed us with strength and chance to complete this study.

Foremost, we would like to pay our cordial gratitude to our venerate and sincerest supervisor of Department Industrial Biotechnology, **Dr. Saadia Andleeb** for her patient guidance, encouragement, and advice she has provided throughout our time as her student. We have been extremely lucky to have a supervisor who cared so much about our work, and who always responded to our queries so promptly despite having a busy schedule. It was a real privilege for us to experience as well as learn from her exceptional scientific knowledge and extraordinary human qualities. Our special regards to my thesis committee member**, Dr. Abdur Rahman,** and for their availability and constructive suggestions. We would also like to extend our gratitude to Principal ASAB, **Dr. Hussnain A. Janjua,** and the esteemed faculty for providing necessary research facilities inthe department.

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Esha Maryam

Shanza Jabeen

Momna Nasir

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LIST OF ABBREVIATIONS

EPM	Elevated Plus Maze Test
WHO	World Health Organization
HMP	Human Microbiome Project
CNS	Central Nervous System
ANS	Autonomic Nervous System
ENS	Enteric Nervous System
HPA	Hypothalamus Pituitary Axis
IBS	Inflammatory Bowel Syndrome
GABA	Gamma-Aminobutyric Acid
SCFAs	Short Chain Fatty Acids
FISH	Fluorescence In Situ Hybridization
PCR	Polymerase Chain Reaction
CUMS	Chronic Unpredictable Mild Stress
MDD	Major Depressive Disorder
USFDA	United States Food And Drug Administration
FDA	Food And Drug Administration
IBD	Inflammatory Bowel Disease
AID	Antibiotics-Associated Diarrhea
CDAD	Clostridioides Difficile-Associated Disease
IACUC	Institutional Animal Care And Use Committee
TSA	Tryptic Soya Agar
EBM	Eosin-Methylene Blue

ABSTRACT

Escalation of antibiotics usage in recent years has imposed serious impacts on physical as well as on mental health. Overuse of antibiotics is known to cause imbalance of gut microbiome profile which in turn can lead to the development of stress and anxiety like behaviors. However, efficacy of restoring disturbed gut microbiome after the overuse of Ciprofloxacin by using probiotic *Bacillus clausii* is not well researched. In this research, we developed an animal model by using 24 BALB/c male mice of weight 25 mg ± 10mg and divided them equally into two control groups; positive control and negative control, and into two experimental groups to study the effect of Ciprofloxacin and *Bacillus clausii* on gut microbiota and behavioral response. In negative control group, mice were provided with appropriate food and saline water for 3 weeks. In positive control group, 135mg/kg/d of antibiotic Ciprofloxacin was administered to each mice for period of 3 weeks. In the first experimental group, mice received antibiotic Ciprofloxacin dosage of 135mg/kg/d and were alongside treated with the probiotic *Bacillus clausii*, with a time interval of three hours between administrations for a period of 3 weeks. In experimental group 2, mice were first administered with antibiotic Ciprofloxacin for 3 weeks followed by treatment with probiotic *Bacillus clausii* for 1 week. After the end of treatment plan, different behavioral tests including Elevated Plus Maze test, Tail suspension test, Forced Swimming test and Marble Burrowing test were performed on mice to examine the effect of Ciprofloxacin and *Bacillus clausii* on neurobehavioral changes in body. Fecal samples were collected after the end of treatment period to analyze and estimate the population of *Bacillus clausii* in gut using Tryptic Soya Agar (TSA) microbial culture testing technique . Results of behavioral tests revealed that experimental group 1 (Ab+pb), showed the most significant results and found to be most mobile among all other groups. In experimental group 2 (Ab with pb), behavioral response of mice was found to be less efficient, showing lesser activity, as compared to the experimental group 1 . Whereas, positive control group (Ab only) displayed the least mobility, demonstrating depressant effect of Ciprofloxacin. In negative control group (Normal saline), mice showed no anxiety and depression like behavior and their behavioral response was found to be consistent with normal

behavior. Microbial culture test results showed that probiotic *Bacillus clausii* was able to sustain most efficiently in experimental group 1 (Ab+pb) in comparison with other groups. These results revealed that administration of probiotic *Bacillus clausii* along with antibiotic mitigated the antibiotic-induced stress. Simultaneous supplementation of probiotic *Bacillus clausii* along with Ciprofloxacin could alleviate the mental stress and facilitate the restoration of gut dysbiosis caused by Ciprofloxacin.

Chapter 1

INTRODUCTION

Antibiotics have revolutionized modern medicine by effectively combating bacterial infections and saving countless lives. However, the indiscriminate use of antibiotics can have unintended consequences on the delicate balance of the gut microbiota, leading to dysbiosis and subsequent stress-related health issues. Antibiotic-induced dysbiosis refers to the disruption of the normal composition and functioning of the gut microbiota due to antibiotic treatment.

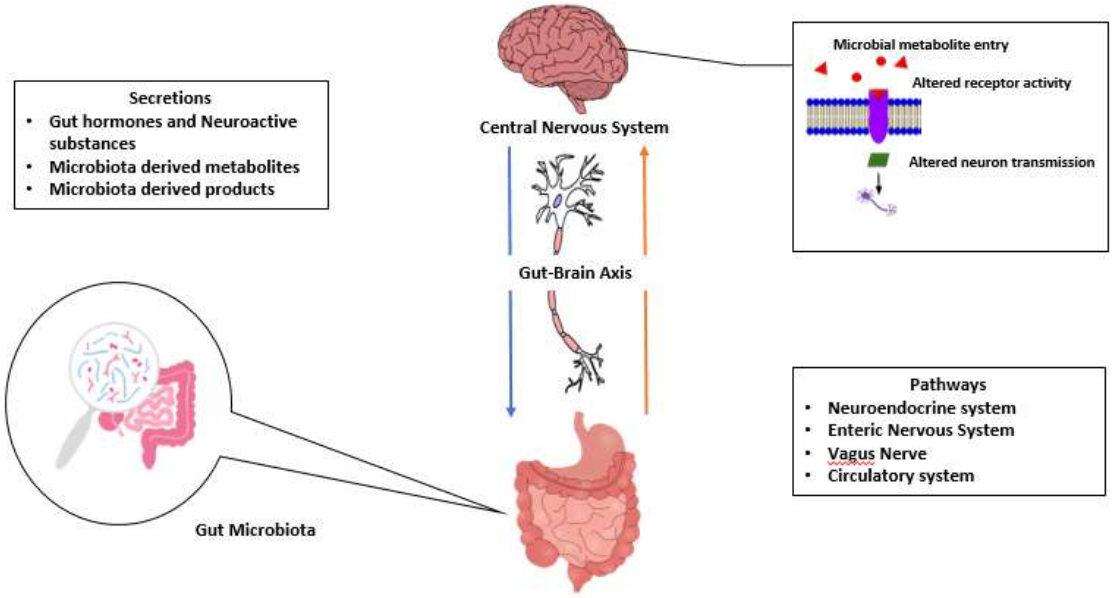

FIGURE 1 GUT MICROBIOTA AND BRAIN AXIS

The gut microbiota plays a crucial role in maintaining overall health, including immune function, digestion, nutrient absorption, and even influencing behavior and mental well-being. When the equilibrium of the gut microbiota is disturbed by antibiotics, it can result in adverse effects such as gastrointestinal discomfort, compromised immune response, increased susceptibility to infections, and even behavioral changes.

Probiotics, which are live microorganisms that confer health benefits when consumed in adequate amounts, have emerged as a potential strategy to counteract antibiotic-induced

dysbiosis and associated stress. Probiotics can restore microbial diversity, enhance the growth of beneficial bacteria, and improve the overall balance of the gut microbiota.

Several studies have demonstrated the efficacy of probiotics in preventing or ameliorating antibiotic-induced dysbiosis in various animal models and human subjects. These beneficial effects of probiotics include the modulation of gut microbiota composition, the production of antimicrobial substances, the strengthening of the intestinal barrier function, and the regulation of immune responses.

However, despite promising findings, the precise mechanisms by which probiotics exert their effects on antibiotic-induced dysbiosis and stress are not yet fully understood. Moreover, the optimal strains, dosages, and duration of probiotic supplementation for different conditions and antibiotic regimens are still areas of ongoing research.

Therefore, the evaluation of the efficacy of probiotics against antibiotic-induced dysbiosis and stress is of significant scientific interest and clinical relevance. Such studies aim to investigate the potential benefits of probiotics in restoring gut microbiota homeostasis, reducing inflammation, normalizing immune responses, and alleviating stress-related symptoms.

By comprehensively evaluating the effects of probiotic supplementation in animal models or human subjects subjected to antibiotic treatment, researchers can provide valuable

insights into the potential mechanisms of action and therapeutic applications of probiotics. This knowledge can contribute to the development of evidence-based recommendations for probiotic interventions to mitigate the negative consequences of antibiotic therapy and improve patient outcomes.

In this study, we aim to evaluate the efficacy of probiotics as an intervention against antibiotic-induced dysbiosis and stress using a well-established animal model. By examining various parameters, including gut microbiota composition, inflammation markers, stress-related behaviors, and physiological well-being, we seek to elucidate the potential benefits and underlying mechanisms of probiotics in restoring gut microbiota balance and ameliorating stress-induced health complications. The findings of this study may have implications for future probiotic interventions in clinical settings and contribute to a better understanding of the role of gut microbiota in maintaining overall health and well-being during antibiotic treatment.

Chapter 2

Literature Review

In modern times, the stress factors affecting people within this interrelatedness and the consequent psychiatric disorders that influence the life quality of human beings have moved to the center of human life itself. World Health Report 2001 shows that depression, among t[these psychiatric disorders, has become the cause of "the largest amount of non-fatal burden globally" (WHO, 2001; Ustun et al., 2004). Current research findings show an intrinsic relation of co-morbidity between chronic medical and psychiatric (especially mood) disorders (Moussavi et al., 2007; Lieshout et al., 2009). For example, in addition to being a prime risk factor for myocardial infarction, it has also been observed to increase the risk of death from cardiac disorders. Also, higher rates of obesity, hypertension, dyslipidemia, metabolic syndrome, and diabetes have been observed in persons with depression than those without depression (Forsythe et al., 2010).

Gut-brain communication

Although microbiology and neuroscience have developed historically as separate fields, recent studies show that microbiota; especially within the gut have a great influence on physiology in general. Large-scale Metagenomics projects, such as Human Microbiome Project have shown the central role that needs to be attributed to microbiota in issues of health and disease (Dinan and Cryan, 2012). The influence of microbiota on CNS has been revealed in several recent studies (Heijtz et al., 2011; Clarke et al., 2012). According to the reports, neuroactive compounds derived from 2 the intestinal lumen can permeate the mucosa; cross the blood-brain barrier; and cause cognitive, psychiatric, and behavioral

disturbances (Wakefield., 2002). Current research has devoted considerable attention to intestinal microbes' role in gut-brain communication. (Figure 1.1)

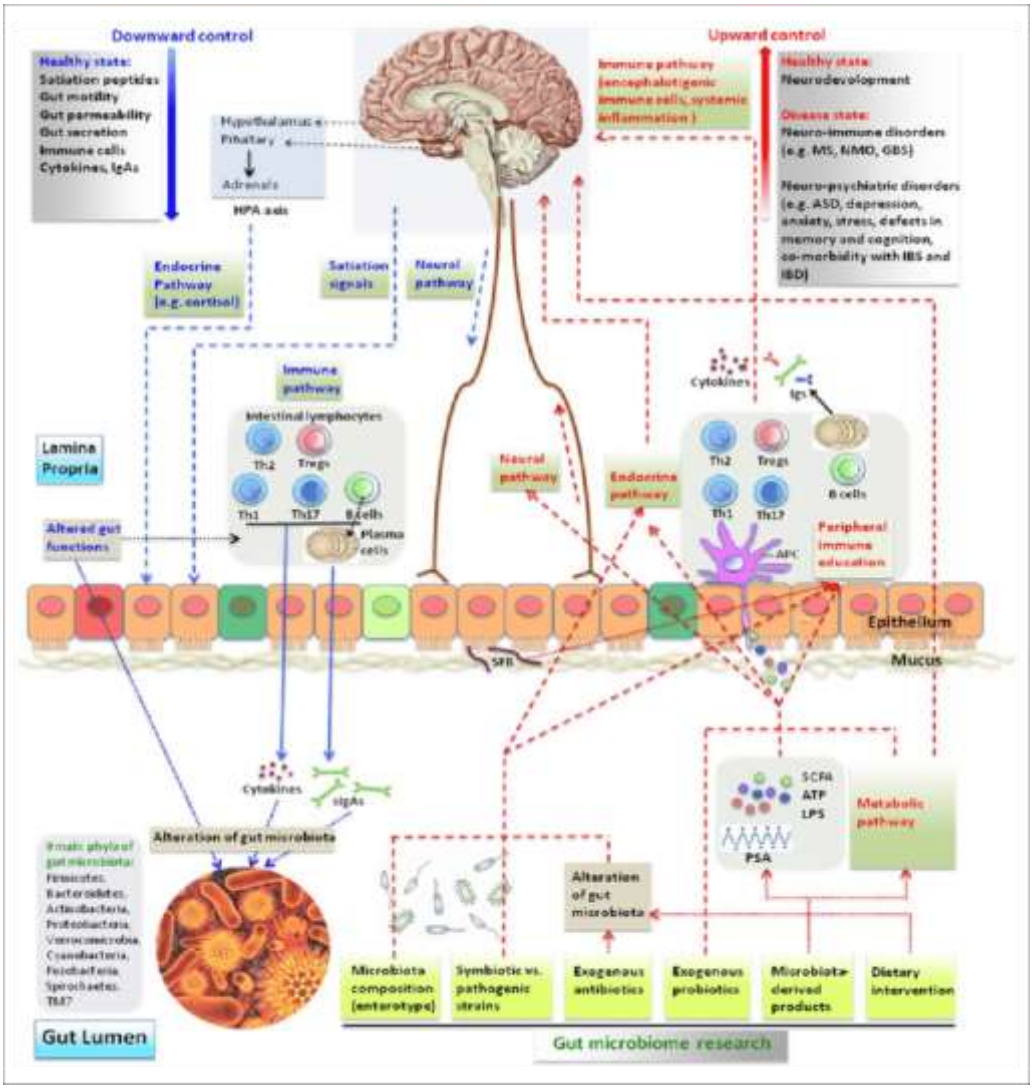

FIGURE 2 MICROBIOME GUT BRAIN AXIS RELATION TO CNS ORDERS

Bidirectional pathways between brain-gut communications

Bidirectional pathways between brain-gut communications have been known to include autonomic nervous system (ANS), the enteric nervous system (ENS), the neuroendocrine system, and the immune system (Figure 1.2). Within this communicative web between CNS and periphery, recent studies have highlighted the importance of 'bottom-up' influence exerted by microbes on CNS, especially the commensal bacteria (Foster and Mcvey, 2013).

By the way, the 'top-down' factors such as anxiety or stress might impair gut functions in such a way that diarrhea, nausea, and discomfort occur (Farmer et al., 2014). Since sporadic changes in dietary habits can cause parallel fluctuations in gut microbiota, they also have an influence on the gut-brain axis and which in turn might

have an influence on behavior including anxiety and depression (Luna and Foster, 2015). The link between the gastrointestinal tract and brain functions has been recognized since the mid-nineteenth century in the work of researchers such as Claude Bernard, Ivan Pavlov, William Beaumont, William James and Carl Lange. In early in 20th century Walter Cannon, the pioneering researcher in gastrointestinal motility studies had pointed to the effects of brain co-processing in the modulation of gut functions.

In current studies, more and more attention is paid to the bidirectional nature of the gut-brain axis which operates through neural, hormonal, and immunological routes, and it is postulated that dysfunction in this interactive communication system can have pathophysiological consequences. Also, the gut itself has a dense neural network which is capable of functioning independently, even after the connection to CNS has been cut off. Within the bidirectional network, while signals from the brain have an impact on the motor, sensory, and secretory functions of the gastrointestinal tract, the gut can influence brain functions, particularly stress regulation areas such as the hypothalamus. (Cryan and Dinan, 2012; Reber, 2012)

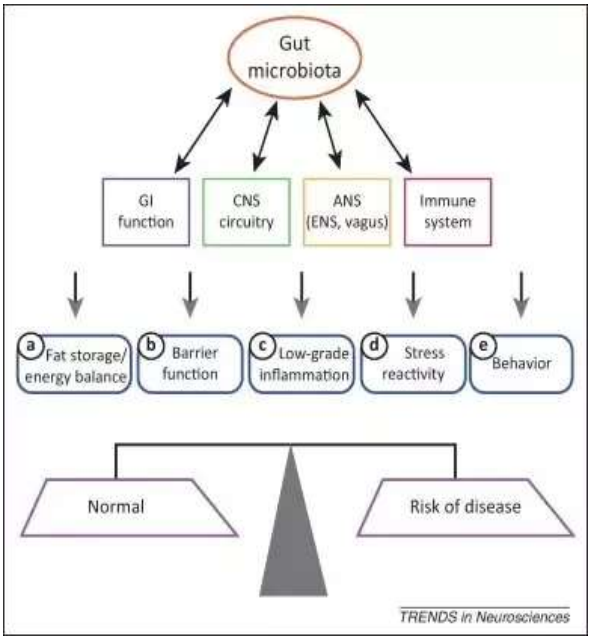

FIGURE 3 BIDIRECTIONAL COMMUNICATION BETWEEN GUT AND BRAIN

Thanks to detailed research on how the brain regulates the enteric nervous system and gastrointestinal functions, it is known that alterations in brain-gut interactions are associated with gut inflammation, chronic abdominal pain syndromes, and eating disorders. Furthermore, it is postulated that alterations in stress response and behavior can be attributed to changes in gut-brain axis functioning (Rhee, 2009). Co- 5 morbidity of psychiatric symptoms such as anxiety, and gastrointestinal disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disorder which occur as a result of gut-brain axis malfunctioning prove its particular importance in pathophysiological studies (Reber, 2012). Intervention on the gut-brain axis is becoming a novel means of developing treatments for a wide range of disorders such as obesity, mood and anxiety disorders, and gastrointestinal disorders such as IBS (Cryan and Dinan, 2012).

GI tract and ENS are modulated by the CNS via sympathetic and parasympathetic branches of the ANS, as well as via the HPA axis. It is said that this kind of influence can change the enteric microbiota in an indirect way by modifying its environment and directly via numerous signaling molecules. (Rhee et al., 2009). Two types of ANS regulate gut functions through the production of bicarbonates, secretion of acid and mucus, regional motility, the permeability of the intestine, maintenance of epithelial fluid, and the mucosal immune response (Figure 4) (Mayer et al., 2000). Except for cortisol-mediated immune regulation, almost all of these functions are affected by sympathetic and parasympathetic neurons on the circuits of the ENS. Furthermore, the enteric microbiota, nutrient delivery rate (such as prebiotics, including certain dietary fibers and resistant starches), gas composition, and other aspects of the luminal environment are expected to be under regional and overall modifications during GI transit (Mayer et al., 2017). Impaired intestinal transit has been related to an overgrowth in the small intestine that is under parasympathetic modulation (Van Felius et al., 2003). In patients with slow transit constipation, a smaller

amount of giant migrating contractions in the colon has been discovered, and in some patients, this is thought to be a likely contributor to the symptoms of IBS as well as constipation. On the other hand, the acceleration of intestinal transit, characterized by a higher number of giant migrating contractions was found in diarrhea cases (diarrhea-predominant IBS (Chey et al., 2001). Besides, factors such as the frequency of food intake, stress, and quality of sleep interfere with the frequency of regular migrations in motor complexes. Furthermore, reduced vagal output to the stomach

and increased parasympathetic output to the large and small intestine are also associated with acute stress (Mayer et al., 2000).

Although there are yet no studies on the outside setting of bacterial overgrowth, the gut transit alterations usually have a higher impact on the composition and organizational structure of gut microbiota in more than one region of GI tract. It has been detected that the size and quality of the intestinal mucus layer, an important habitat for the biofilm microorganisms, is affected by ANS-mediated modulation. In this habitat, almost all-enteric microbiota reside (Macfarlane and Dillon, 2007). Also, ANS can modify the epithelial mechanisms of the immune system involved in the activation by the gut. This activation can occur in two ways: either directly through 7 the response modulation of the gut immune cells such as mast cells and macrophages to luminal bacteria with the antimicrobial peptides (Alonso et al., 2008) or indirectly by modifying the access of the luminal bacteria to immune cells of the gut. For instance, many of the preclinical studies have demonstrated that, in the intestinal mucosa, stressful stimuli can aid the translocation of luminal organisms, increase the permeability of the intestinal epithelium, and stimulates an immune response (Groot et al. 2000; Keita and Soderholm, 2010).

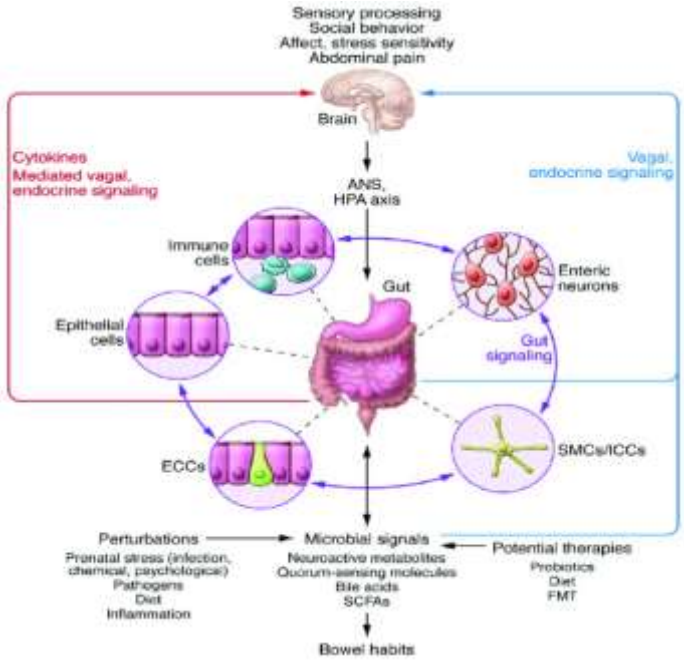

FIGURE 4 GUT BRAIN BIDIRECTIONAL INTERACTION

Modulation of gut microbiota by host-derived signaling molecules

CNS-induced changes in the gut composition are modulated by neuroendocrine and neuronal signaling. Signaling molecules, including but not limited to catecholamines, GABA, dynorphin, serotonin, and cytokines; may also be released into the gut lumen by neurons, enterochromaffin, and immune system-related cells (Lyte and Freestone, 2010; Lyte et al. 2013; Yang et al., 1992; Santos et al., 1998). Catecholamines are an especially well-investigated case of signaling molecules that enable direct host-to-microbe signaling. Various kinds of stressors can increase plasma and local levels of catecholamines such as norepinephrine and also raise luminal levels in the gut (Alverdy et al., 2000; Hughes and Sperandio, 2008). Some of the pathogens can change their spawning activity in response to external catecholamines in vitro (Lyte et al., 2004). For example, the proliferation of several strains of enteric pathogens can be triggered by norepinephrine. Similarly, the virulent properties of Campylobacter jejuni can be increased by norepinephrine (Hughes et al., 2008; Cogan et al., 2007). Still, the influence of catecholamines on nonpathogenic organisms and other microbial signaling molecules on gut metabolic activity and microbiota composition in diseased and healthy individuals is not known (Mayer et al., 2017).

Microbe-to-host signaling by microbial-generated signal molecules

Upon establishing the fact that the gut microbiota can communicate with ENS (Frosythe and Kunze 2013) and the brain, some of the microbe-generated signal molecules have been characterized (Figure 1.4). Quorum-sensing molecules, such as metabolites and neurotransmitter homologs, are used by microbes to communicate with each other. These molecules are also recognized by host cells and may influence immune cells, enteroendocrine cells, and nerve endings in the gut (Rhee et al., 2009).

During the immune response, metabolites produced by gut microbiota, including bile acids, SCFAs, and neuroactive substances such as tryptophan precursors, GABA, serotonin, catecholamines, and cytokines are released. They can signal the host via receptors on local cells within the gut (Chey et al., 2001; Bailey et al., 2011). Moreover, a high amount of metabolites found in the circulation 9 originates from gut microbiota. These metabolites theoretically constitute a basis for a vast gut microbiota–to–brain signaling system (Wikoff et al., 2009).

Fermentable carbohydrates, including acetate, butyrate, and propionate reach into the colon and are transformed into SCFAs (well-known examples of microbe-derived

metabolites). Main SCFAs have a number of physiological effects, containing reduction of food intake, modulation of lymphocytes, improvement of glucose tolerance, neutrophil functions, and activation of epithelial cell signaling pathways (Cummings et al., 1987; Nepalska et al., 2012; Cani et al., 2013). For instance, a diet supplemented with Bifidobacterium breve

caused high fatty acid concentrations in the brain; yet the mechanisms lay beneath these effects are not known yet (Wall et al. 2012). The microbiota can influence relations between the gut and the nervous system through multiple different mechanisms (Figure 1.5). Without taking the succession of events causing a state of dysbiosis in a particular disorder into consideration, the bidirectional communication between the gut and brain is likely to be affected by alterations in the microbial composition. Such effects may emerge early in life and affect the development of the nervous system. The HPA axis, and the brain's interaction with the intestine; in adults, may act on completely developed circuits (Figure 1.4)(Cryan et al. 2012; Forsythe and Kunze 2013; Bercik et al., 2012). Perhaps, some of these signaling mechanisms operate in the presence of an intact epithelium (e.g., by vagal signaling). However, the effects are likely to be enhanced and changed in the context of increased intestinal permeability induced by mucosal inflammation or stress (Soderholm et al., 2001; Hsiao et al., 2013; Leclercq et al., 2014).

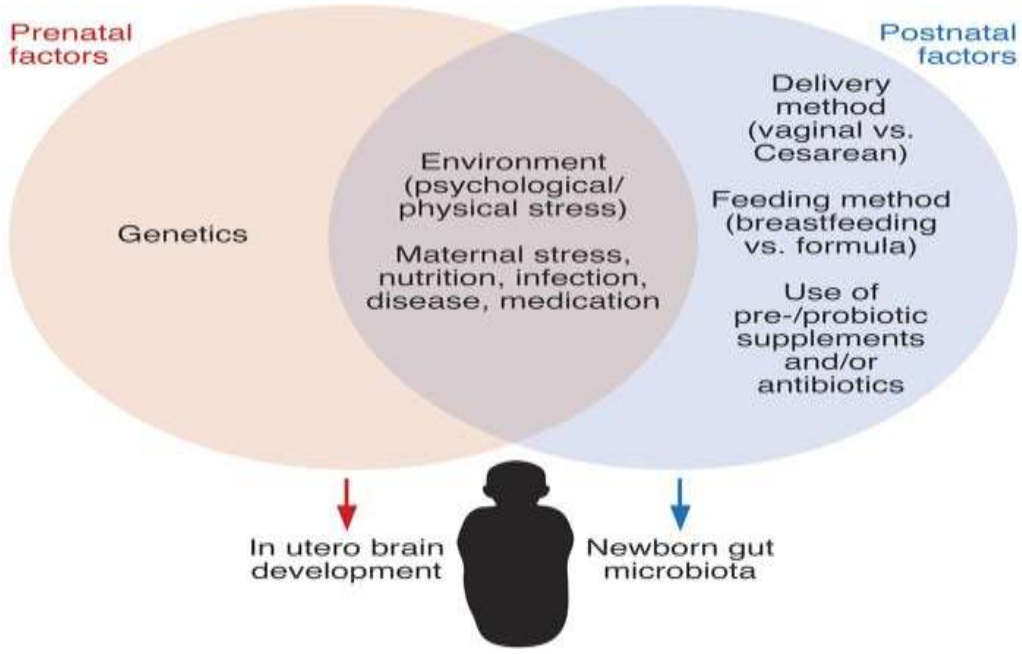

FIGURE 5 INFLUENCE ON GUT MICROBIOTA/AXIS IN THE PERINATAL PERIOD

The microbial profile at perinatal period

The gut is sterile at birth. The human gut plays a role in metabolic and protective functions, ranging from creating important vitamins and amino acids to protecting cells that line the gut from injury. Right after birth and within the first year, bacterial colonization occurs which is a process that shows immense temporal and individual fluctuations of bacterial populations. The bacterial profile established during this period, which in general has a maternal signature, converges with that of the adult, and it has been observed that despite drastic influences by disease, infections, stress, and diet, this intestinal microbiome profile tends to revert to that established in infancy. However, "the core microbiota of the aged individual is distinct from that of the younger adults" (Cryan and Dinan, 2012).

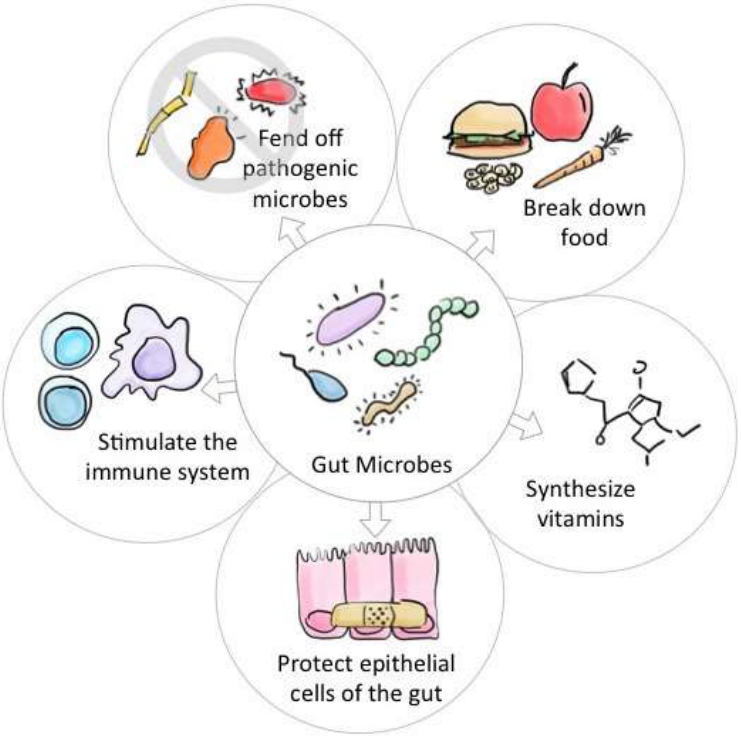

FIGURE 6 MAIN FUNCTIONS OF HUMAN GUT MICROBES

The complexity of microbial communities did not allow the application of traditional approaches that rely on isolating microbes in cultures to create a complete map of the intestinal microbiome. This culture-based analysis is only suitable for microbiota that can be cultivated. However, recent developments in molecular microbiology technologies have enabled genetic analysis of complex microbial populations

(Forsythe et al., 2010). These culture-independent techniques include sequencing, genetic fingerprinting, fluorescently oligonucleotide probes (FISH), quantitative PCR as well as metagenomics approaches (Archie and Theis, 2011).

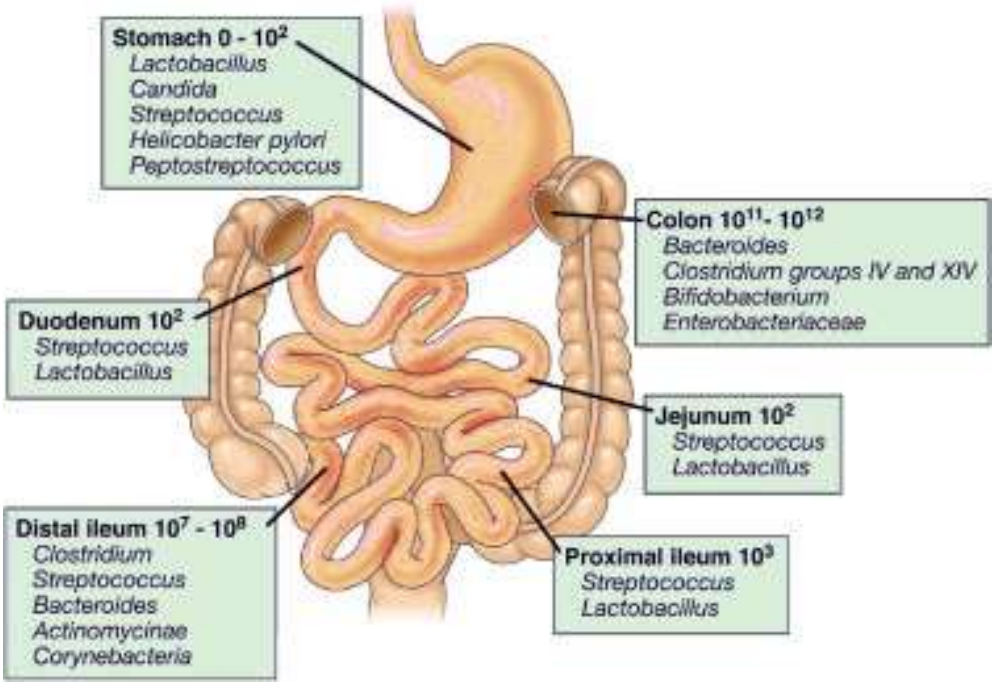

FIGURE 7 DISTRIBUTION AND ABUNDANCE OF BACTERIA IN HUMAN GASTROINTESTINAL TRACT

Dysbiosis of Gut Microbiota and Mental Stress:

Human gut microbiota is comprised of huge variety of approximately 40,000 bacterial species and around 1800 different phyla, and their role and impact has been studied and associated in different human diseases (Viswanath & Q. Parker-Actlis, 2020) . The imbalance of gut microbiota is called dysbiosis. It is important to note that gut flora varies between humans due to the different lifestyles, eating habits, genus, age, and ethnicity. Gut flora actual functional properties and characterization remains unclear but a slide change or modification in gut microbiota (dysbiosis) can put body under stress. The exact mechanism through which gut microbiota modulates the brain activity and causes mental stress is still unknown. However, much research has found evidence that disturbance in gut microbiome profile induce depression and anxiety like behaviors. The microbial analysis of fecal samples of rats put under artificially induced stress i.e., Chronic Unpredictable Mild Stress (CUMS) has shown that

beneficial bacteria decline in obvious amounts and causes dysbiosis, thus impairing cognitive and emotional behavior in rats (Viswanath & Q. Parker-Actlis, 2020). Another study has shown that bacterial transplantation from patients with
Major Depressive Disorder (MDD) has caused depression like behaviors in rats. Comparative analysis of fecal samples of patients with MDD and the people with healthy gut microbiome showed visible difference between microbial population of gut microbiota (Sublette & Uhlemann , 2019).

Interlink of Antibiotics and Mental Stress

Antibiotics are the medications that are prescribed for various bacterial infections. They are in clinical practice from years. However, the overuse of antibiotics due to accessibility and availability, have reportedly caused adverse side effects in humans. Antibiotics not only kills the pathogenic bacteria, but also harms the beneficial bacteria, thus disturbing the normal gut microbiota of body. Altered gut microbiota composition is known to cause under secretion of neurotransmitters such as serotonin, dopamine etc. that play their role in mood defining and cognitive behavior (Chen & Chen, 2021). A recent study has shown that in a survey, 94 patients who took fluoroquinolones reported the following psychiatric side effects: anxiety disorder (72%), depression (62%), insomnia (48%), panic attacks (37%) and cognitive impairment (33%) (Bennet & Ablin, 2021). Another study has shown that antibiotics like penicillin and fluoroquinolones accelerated the risk of depression, psychosis and anxiety among patients (Boursi, 2015).

Introduction to Ciprofloxacin

Ciprofloxacin is an antibiotic agent that belongs to the class fluoroquinolones which are used to treat bacterial infections such as Pneumonia and Urinary tract infections. Ciprofloxacin is a second-generation fluoroquinolones antibiotic agent, and its main antibacterial activity is against *Pseudomonas aeruginosa*. It was first patented by Bayer A.G. in 1983 and later it was approved by United States Food and Drug Administration (USFDA) in 1987. Since then, it has become a commonly prescribed medication or various infections. Gram-negative and Gram-positive bacteria are both susceptible to its broad-spectrum action. It is commonly used to treat respiratory tract infections, urinary tract infections, skin and soft tissue infections, bone and joint

infections, gastrointestinal infections, and sexually transmitted infections. Additionally, ciprofloxacin is employed as a prophylactic agent for individuals exposed to anthrax (Brubaker et al., 2018; Xu et al., 2017).

Mechanism of Action of Ciprofloxacin

Ciprofloxacin exerts its action by inhibiting two essential bacterial enzymes; bacterial *DNA gyrase* and *Topoisomerase IV* enzymes, which are necessary for DNA replication, transcription, repair, and recombination. It binds to the A subunits of enzyme DNA gyrase, forming a stable drug-enzyme complex, thus preventing the negative supercoiling into the bacterial DNA during replication phase. *Topoisomerase IV* aids the disintegration of integrated DNA strands during the process of cell division of bacteria. Ciprofloxacin binds to the B subunits of *Topoisomerase IV and* inhibits the separation of daughter DNA molecules. Thus, Ciprofloxacin inhibits the manufacture of bacterial DNA by targeting these enzymes, which prevents bacterial growth and ultimately results in cell death.

Ciprofloxacin and its Clinical uses:

Due to its potency against a variety of bacterial infections, the fluoroquinolone antibiotic ciprofloxacin, which has a broad spectrum of activity, is frequently employed in clinical practice. Targeting common respiratory pathogens like Streptococcus pneumoniae, Haemophiles influenzae, and Moraxella catarrhalis, it is widely recommended for respiratory tract infections such pneumonia, bronchitis, and sinusitis. Escherichia coli, Klebsiella pneumoniae, and Staphylococcus saprophyticus are just a few of the Gram-positive and Gram-negative bacteria that can cause urinary tract infections that are treated with ciprofloxacin.

Additionally, it exhibits effectiveness in the treatment of bone and joint infections in addition to skin and soft tissue infections like cellulitis and abscesses. Ciprofloxacin can also be used to treat sexually transmitted infections like gonorrhea as well as gastrointestinal infections such infectious diarrhea and typhoid fever. Additionally, ciprofloxacin is utilized as a preventative medicine in those who have been exposed to anthrax, as it has been shown to be effective against Bacillus anthracis, the causative organism.

Impact of Ciprofloxacin on Gut microbiome diversity:

Ciprofloxacin, like other antibiotics, can impact the gut microbiome profile and thus resulting in gut dysbiosis. It mainly targets pathogenic bacteria but can also affect the beneficial bacteria which plays an important role in maintaining and optimizing the gut health and its microbiome diversity. According to studies, using ciprofloxacin can lead to a decline in the diversity and number of beneficial bacteria like lactobacilli and bifidobacteria while also possibly promoting the growth of pathogenic bacteria like Clostridium difficile. Dysbiosis, an imbalance in the microbial population, can be caused by these alterations in the gut microbiome and may have effects on gastrointestinal health and general wellbeing, a microbial population imbalance that could affect the general health and wellbeing of the gastrointestinal tract. A study has found that fluoroquinolones can reduce gut microbial diversity by 25%.

Impact of Ciprofloxacin on Neurological functions:

Ciprofloxacin has been used widely for many years in clinical practices and its side effects have been reported often. The most reported side effects are sleeplessness, diarrhea, nausea, vomiting, dizziness, and headaches (Heather Holmstrom, 2013). The US Food and Drug Administration (FDA) has given caution about the adverse side effects of usage of Ciprofloxacin and other Fluoroquinolones on the Central Nervous System (Heather Holmstrom, 2013). According to FDA recommendation, drugs like Ciprofloxacin should not be advised as first-choice antibiotics for respiratory or urinary tract infections. In severe cases, the side effects like hallucinations, suicide attempt, paranoia, and psychosis have also been reported so far (Heather Holmstrom, 2013). Approximately, a total of 9.3% cases has the reported side effects of Ciprofloxacin. The intensity of symptoms depends on the antibiotic dosage and its duration.

Introduction to Probiotics:

Probiotics have caught significant importance in recent years due to their importance in optimizing and maintaining normal body functions. These are the living microorganisms that provide benefit to their host when are consumed in adequate amounts. They are normally found in gastrointestinal tract and include some types of yeast and mostly bacterial species such as *Lactobacillus* and *Bifidobacterium* species.

The idea of probiotics came from Russian scientist Elie Metchnikoff in early 20th century when she observed that people who intakes fermented dairy products have improved health and longevity. This idea generated research on probiotics, and it has been continued since then. Probiotics benefit their host via various mechanisms. They improve the gut brain axis, thus inhibiting the growth of pathogens. Besides this, they can lower the pH and y also provide short-chain fatty acids and antibacterial compounds that will develop the unsuitable conditions for other pathogens and will maintain the gastrointestinal tract health (Williams, 2010). Probiotics can treat the antibiotics induced diarrhea and its strong evidence is found in pouchitis and rotavirus (Fernando Rizzello, 2012) . More research is needed to study the effect of probiotics in antibiotics induced neurological changes.

Role of Probiotics in restoring Gut microbiome:

Probiotics are known to improve gut health by optimizing the microbial diversity disturbed due to some event. The imbalance of gut microbiota is called dysbiosis. It is important to note that gut flora varies between humans due to the different lifestyles, eating habits, genus, age, and ethnicity. Gut flora actual functional properties and characterization remains unclear but a slide change or modification in gut microbiota(dysbiosis) can put stress on body. Several diseases are known to be caused by disturbed gut microbiome such as Inflammatory Bowel Disease (IBD), Inflammatory Bowel Syndrome (IBS). Recent research has suggested that probiotics can restore the gut microbiome and can ultimately reverse the pathogenesis of diseases. The antibiotics-associated diarrhea (AID) which is reported in total 30% of cases can be reversed by treating it with probiotics (Lal & Kumar, 2019) .

Introduction to *Bacillus clausii:*

Bacillus clausii is a probiotic that benefits human health. Its key characteristics include the ability to form spores; the resulting tolerance to heat, acid, and salt ensures safe passage through the human gastrointestinal tract with no loss of cells. When environmental conditions are harsh, spore-forming bacteria undergo a complex developmental process in which the bacterial cell differentiates into a spore that can indefinitely survive in the absence of water, nutrients, extremes of temperature, pH, ultraviolet radiation, and noxious chemicals. When favorable environmental

conditions return, the spores germinate into vegetative cells that can grow and reproduce. (Current Progress and Future Perspectives on the Use of Bacillus clausii, 17 June 2022). *Bacillus clausii* and *Bacillus licheniformis* have been isolated from healthy human adult feces, indicating their ability to survive passage through the gastrointestinal tract.

Bacillus clausii as a probiotic

Probiotic bacteria and dietary supplements have the potential to prevent or reverse imbalances in the gut microbiota caused by antibiotics. Clinical studies have shown that probiotics can help prevent antibiotic-associated diarrhea (AAD) and, to a lesser extent, Clostridium difficile-associated diarrhea (CDAD). They have also been found to improve the eradication rates of H. pylori. In children, specific probiotics like Saccharomyces boulardii and Bacillus clausii have been shown to reduce the frequency and duration of acute diarrhea. Bacillus clausii not only has antimicrobial properties against various bacteria but also modulates the immune system, which contributes to its reported benefits as a probiotic.

Due to their natural resistance to antibiotics and the high quality of certain probiotic formulations, B. clausii strains have been used alongside antibiotics to minimize gastrointestinal side effects. For example, the strains Bacillus clausii O/C (CNCM I-276), N/R (CNCM I-274), SIN (CNCM I-275), and T (CNCM I-273) marketed as Enterogermina® by Sanofi, have been safely and effectively used in humans for many years. These strains, derived from a penicillin-resistant strain called B. subtilis ATCC 9799, were initially classified as B. subtilis but were reclassified as B. clausii in 2001. They have been available as over-the-counter medicine since 1999.

B. clausii strains have been extensively studied and possess beneficial physiological properties such as tolerance to heat, acid, and bile salts, improvement of gut barrier function, broad-spectrum antibiotic resistance that cannot be transferred to other species genetically, and the ability to synthesize vitamins.

Antibiotic Resistance in *Bacillus clausii* :

The contamination of aquatic environments by tetracycline antibiotics (TCs) is an increasingly pressing issue. The antibiotic resistance of *B. clausii* strain T has been leveraged to remove antibiotics tetracycline, oxytetracycline, and chlortetracycline

from aquatic environments. Vegetative cells of the *B. clausii* strains T and O/C remove a mix of antibiotics cefuroxime, cefotaxime, and cefpirome from the culture medium.

Antibiotic resistance coupled with the proven inability for this resistance to be transferred to other bacteria is a positive safety attribute of a probiotic. It enables the probiotic to be used concomitantly with antibiotic treatment—one of the contexts in which the gut is likely to be stripped of its natural flora and in need of being re-populated with beneficial bacteria. Therefore, clinicians need to be aware of the antimicrobial resistance profiles of commercially available probiotics.

The vegetative cells of the *B. clausii* strains, O/C, N/R, SIN, and T, are resistant to different degrees to different antibiotics. All strains are fully resistant to erythromycin, azithromycin, clarithromycin, spiramycin, clindamycin, lincomycin, and metronidazole; each strain displays a slightly different resistance profile to some of the other tested antibiotics.

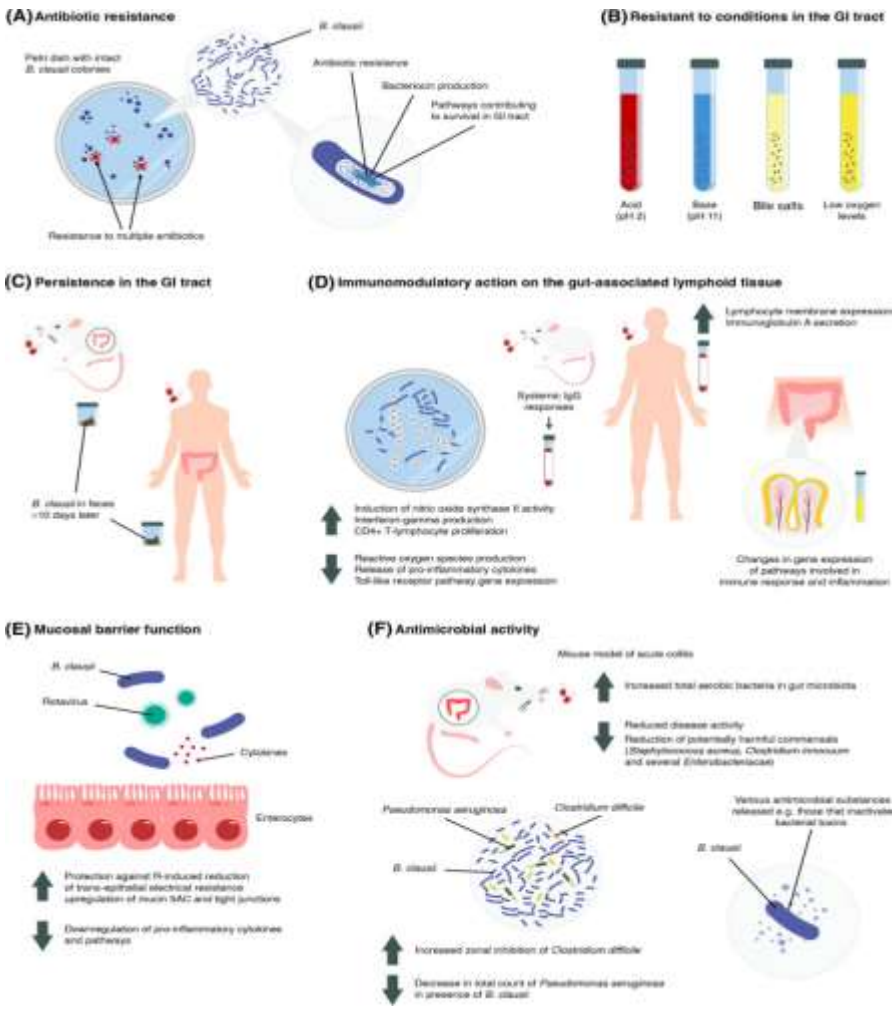

FIGURE 8 *B. CLAUSII* ANTIBIOTIC RESISTANT STRAIN

B. clausii in treating Gastrointestinal Disorders

In a mouse model, the in vivo effects of B. clausii (O/C, N/R, SIN, and T) supplementation on mild acute colitis and gut microbiota composition were recently investigated. Along with a significant reduction in colitis disease activity compared to placebo (days 2-5), B. clausii was linked to changes in the gut microbiota, including a significant increase in total aerobic bacteria and significant decreases in potentially harmful commensals such as Staphylococcus aureus, Clostridium innocuum, and several others.

Bacillus clausii for Gastrointestinal Disorders: A Narrative Literature Review, 2022 August 26). Furthermore, when Pseudomonas aeruginosa was cultivated with B. clausii in vitro, the total count of Pseudomonas aeruginosa was significantly reduced.

These findings point to modification of gut microbiota composition as one possible method.

Chapter 3

Methodology

Habituation of mice:

24 balb c mice will be used were used (NIH, Islamabad) The rats were housed in 4 cages in an ASAB animal house maintained at a constant relative humidity (50%-60%) under 12-h light–dark cycle. Mice received free access to drinking water (replacement at 3-day intervals) and food during experiments.

Experimental design

24 mice will be acclimatized for 1 week before the start of the experiment. They will be then randomly divided into four groups (n =6 rats per group), control (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic (AB-Prob), and antibiotic plus probiotics (AB + Prob). The application of ciprofloxacin for one week was meant to induce dysbiosis in the rats, and one-week probiotic treatment with *Bacillus clausii* was to test the effects of probiotic on gut dysbiosis recovery. Ciprofloxacin was dissolved in phosphate-buffered saline, and the antibiotic dosage (135 mg/kg/d per mice by oral gavage twice daily). The probiotic strain was prepared and given by oral gavage (2.5 109 CFU/day [14] in a sterilized saline solution twice daily). For the AB-Ctrl group, ciprofloxacin was administrated for 3 weeks, followed by saline treatment for 1 week. The AB-Prob group was given the probiotic instead of the saline for 1 week. For the AB + Prob group, probiotic was given three hours after ciprofloxacin administration for 1 week, followed by probiotic treatment for 1 week. The control group was given an equivalent volume of sterilized 0.85% saline solution instead of antibiotic or probiotic, respectively. Fecal samples were collected after the antibiotic and probiotic treatment from each group. Changes in the gut microbiota and colonies of *Bacillus clausii* were compared between different treatment groups after the treatment plan.

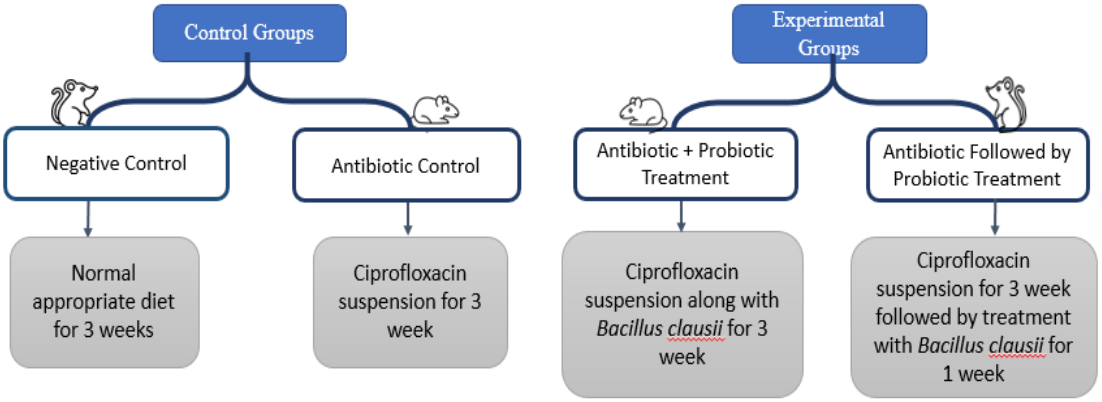

FIGURE 9 DIVISION OF MICE INTO DIFFERENT STUDY GROUPS

Isolation of Probiotics

Four strains of probiotic *Bacillus clausii* **O/C, N/R, SIN,** and **T** are commercially available in the form of proiotic Enterogermina which is used in the treatment of

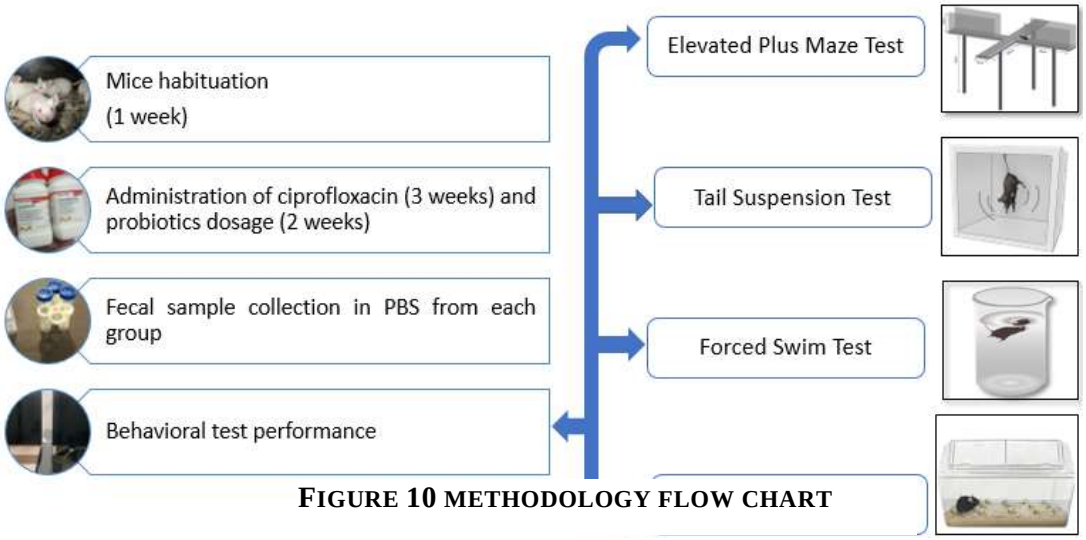

FIGURE 10 METHODOLOGY FLOW CHART

diarrhea. 100 µl of probiotic supplementation was defined for administration to mice during probiotic treatment period.

Treatment Plan

Following treatment plan was designed to administer antibiotic and probiotic dosage to study and examine the effects of Ciprofloxacin and *Bacillus clausii* on neurobehavioral changes in mice.

	Negative Control Group	Antibiotic Control Group	Treatment Group 1	Treatment Group 2
1ST Week	Habituation	Habituation	Habituation	Habituation
2nd Week	NS	Ab	Ab	Ab + Pb
3rd Week	NS	Ab	Ab	Ab + Pb
4th Week	NS	Ab	Ab	Ab+ Pb
5th Week	-	-	Pb	-

FIGURE 11 TREATMENT PLAN FOR THE MICE

Performance of Behavioral Tests

Elevated Plus Maze test

The EPM is another apparatus that has been used for behavioral assays and to measure memory function. The maze is composed of 2 open and 2 closed arms. The length of each open and closed arm is 30 cm and the width is 5 cm. The height of the walls of the closed arms is 20 cm. The central area between the 4 arms is 5 cm^2. The maze was placed on a stand that elevated about 30 cm from the floor. Each mouse was placed in an open arm at the start of the training session. The mouse was allowed to explore the apparatus for 5 min and then returned to its cage. After 3 h, each mouse was placed in the same location where it will be placed during the training session. The time spent in the open arms was measured using a stopwatch. The animal was considered to have entered a closed arm when all its legs were in the closed arm. A video camera was placed above the maze to record each session by the mouse in the open arms and closed arms to score and analyze.

Tail suspension test

The mice tail clamping test is commonly used to assess pain sensitivity, where the tail withdrawal is an indicative of pain response. The test also demonstrates to evaluate the analgesic effects of various substances.

The mice were gently restrained, and their tails were clamped using a specially designed clamp. The pressure applied to the tail was standardized to ensure

consistency across experiments. The mice were monitored for their tail withdrawal response, which was recorded as an indicator of pain sensitivity. Prior to testing, the mice were acclimated to the experimental environment for a certain period of time to minimize stress and anxiety. The duration of the tail clamping was carefully controlled to avoid causing tissue damage or excessive discomfort to the animals.

Different treatments or interventions, such as the administration of analgesic drugs, were often evaluated by observing changes in the tail withdrawal response during the test. The data collected from the test were analyzed to determine the analgesic efficacy of the interventions. Overall, the mice tail clamping test provided valuable insights into pain sensitivity and the effectiveness of analgesic interventions in experimental studies.

Swimming test

In that test, animals were placed in a cylinder tank or another IACUC-approved container* that was partially filled with water, making it impossible for them to escape. The time they spent immobile or floating was recorded, excluding any time spent swimming or attempting to climb the walls. The immobility time was considered a measure of behavioral despair or learned helplessness. The testing was conducted under normal light conditions, and the animals were given a minimum of 60 minutes to acclimate to the test room before the test began. If sudden loud noises could startle the animals (depending on the specific laboratory environment), using a white noise generator was recommended. The Plexiglas container was filled two-thirds of the way with water, maintained at a room temperature of 24°C ± 1°C. Non-toxic agents could be used to color the water. The temperature was checked before each test using an infra-red or glass thermometer. Each mouse was gently inserted into the water by its tail for a 6-minute testing session. However, only the final 4 minutes were analyzed, as nearly all mice would swim continuously in their attempts to escape during the first 2 minutes. The mice were closely observed throughout the test, and if they were in danger of drowning or unable to keep their head above water (which was very rare in a 6-minute test), they were immediately removed.

After the test was completed, the mice were taken out of the tank and placed in their home cage, where they were monitored for 10 minutes. It was expected that the mice would be active and begin grooming themselves to remove excess water. If grooming

behavior was delayed or if the mice appeared hunched, they could be placed in a clean cage with the bottom half on/off a heating pad (37°C) for 10-15 minutes or gently dried with a clean paper towel.

Marble burrowing test

Marble burying is commonly used to test anxiety, where burying is positively correlated with anxiety. The test also demonstrates to analyze repetitive behavior.

Two days prior to training, two glass marbles were placed into the animal's home cage to prevent neophobia during testing. Training and testing were conducted in a new home cage (equal size, filled with 3 cm wood chip bedding, covered with a wired cage lid), under

dimmed light. On the training day, animals were allowed to freely explore the testing cage without marbles for 15 min for habituation. On the testing day, approximately 24 hours after training, 15 marbles were evenly placed in the test cage to ensure equal and consistent positioning of the marbles. Animals were exposed to the marbles for 15 min while being videotaped. The manually evaluated parameters were latency to start burying and the number of buried marbles.

Estimation of culturable microbial diversity

We performed cultural test to estimate the microbial diversity in the mice and to confirm the presence of bacillus clausii. We cultivated the fecal sample collected from mice in general media (EPM) and for more specific results decided to progress toward a more selective media tryptone soya agar (TSA). Lastly, we performed gram staining to confirm the nature of microbes in the media.

Chapter 4

Results

Results of microbial cultural tests

In results of cultural tests the colonies in the general media were merged together we decided to progress toward more selective media. The figures below represent the results of selective and general media.

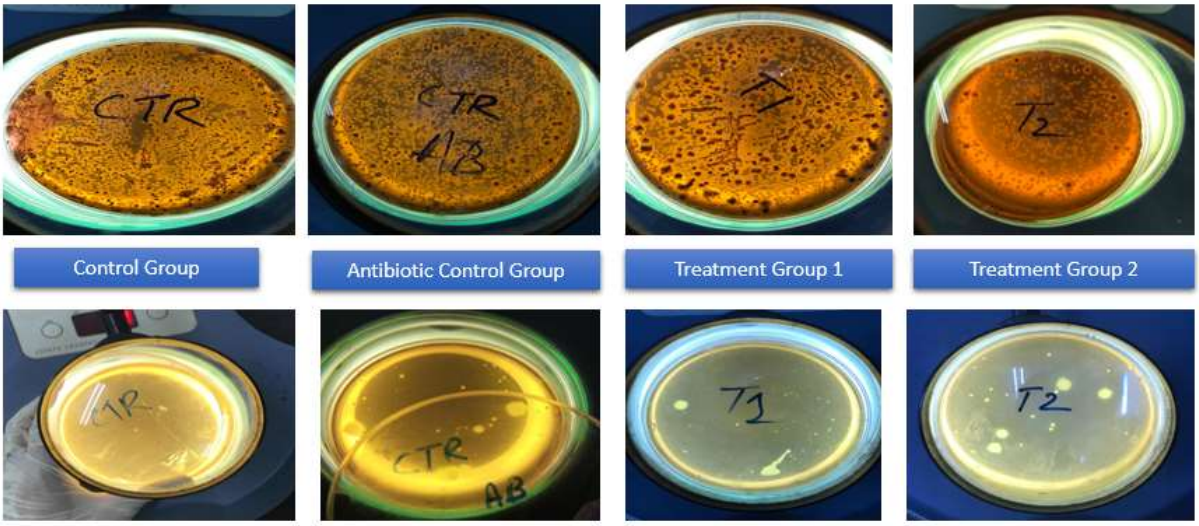

Figure 12. Estimation of culturable microbial diversity

In order to confirm the nature of microbes in the selective media we have performed gram staining. From the results of gram staining it is apparent that the control, antibiotic control, and treatment 1 group we have more gram negative bacteria which appear to be pink in color and in control group there is minute amount of gram positive as well which is evident in the diagram. Due to character specificity of selective media for gram positive bacteria the retention of purple stain in treatment group 2 indicate the successful colonization of bacillus clausii. Because of the successful colonization of bacillus clausii the stress in these mice were reduced as evident in the results of behavioral tests.

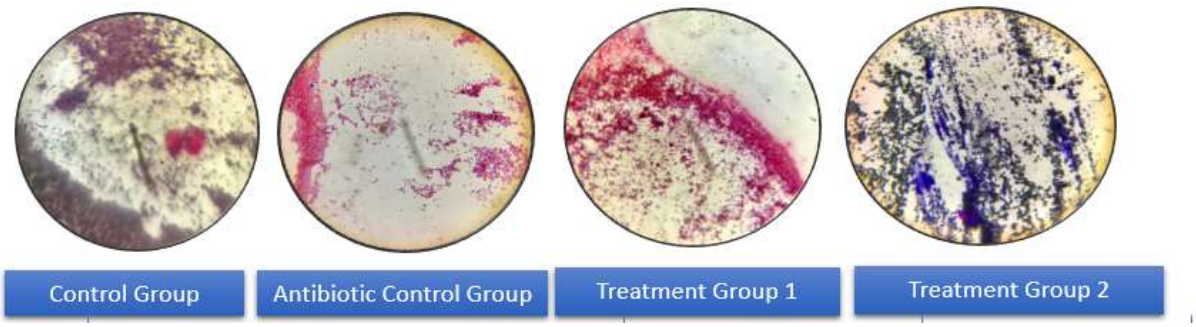

Figure 13. Results of Gram staining to confirm the nature of microbes

Results of Behavioral tests

Forced Swimming Test (FST) Results

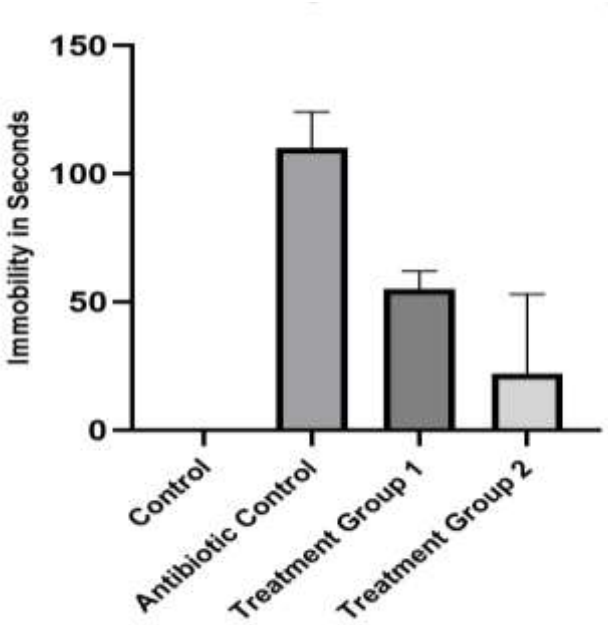

Figure 14. Graphical representation of forced swim test results

In the graph above, Maximum immobility is shown in the antibiotic control during swimming. This indicates that the mice who have taken antibiotics only do indeed show symptoms of depression which is directly related to reduction in mobility. However, after treatment with probiotics the immobility time is reduced slightly in the T1 group which are given antibiotics after one week but in the T2 group which are given probiotics every day, show remarkably better mobility than the antibiotic control in both tests.

Tail suspension test results

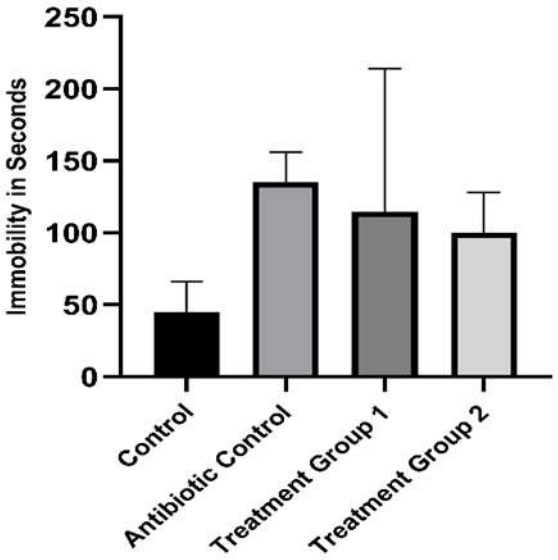

FIGURE 15 GRAPHICAL REPRESENTATION OF TAIL SUSPENSION TEST RESULT

In tail suspension test results the antibiotic control group spent highest time immobile indicating depressed behavior. Treatment group 2 group spent less time immobile as compared to treatment group 1 while control group was the least immobile.

Elevated plus maze test:

In the results of the antibiotic control group has a much higher ratio of time spent in the closed/open arm indicating more anxiety. On the other hand, the treatment 1 and treatment 2 group tend to almost same values as control.

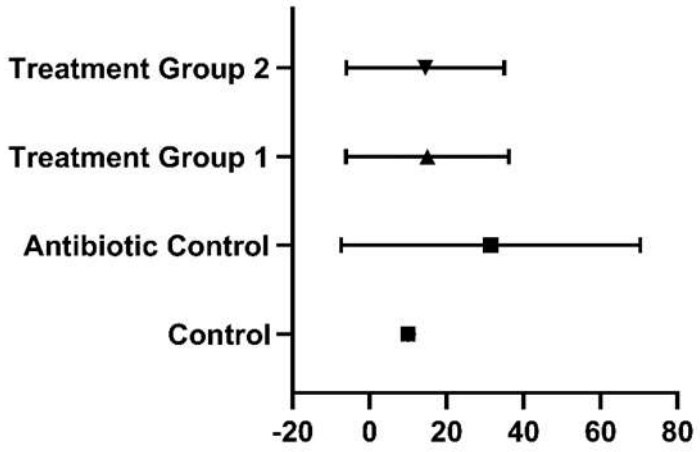

Figure 16. Graphical representation of elevated plus maze test results.

Marble burrowing test results:

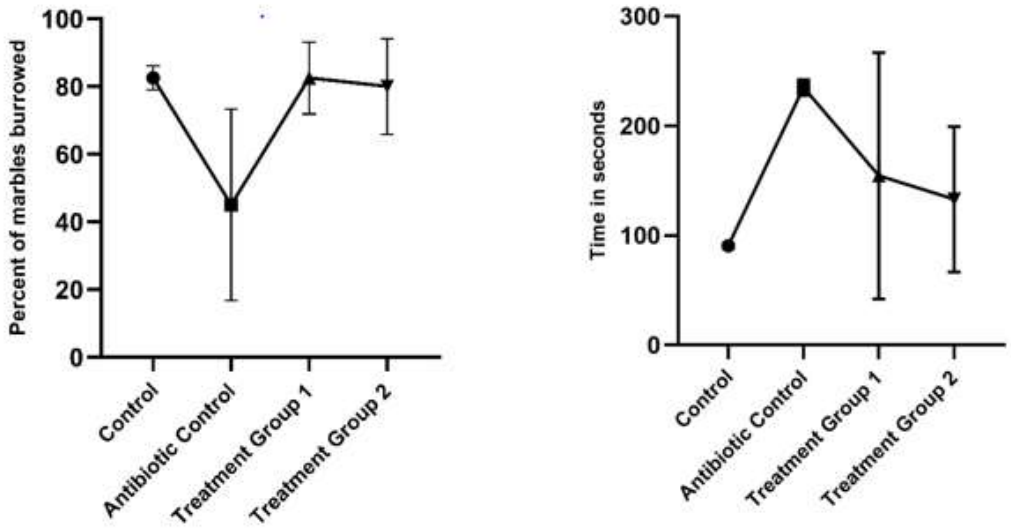

Figure 17. Graphical representation of marble burrowing test results.

The mice in the treatment groups showed more burrowing activity than the antibiotic control group. The overuse of antibiotics leads to mice with more stress resulting in a lesser number of marbles buried and more time taken to interact with the marbles. The treatment group 2 in this case, are observed to reduce the effect of stress caused by the antibiotic.

Chapter 5

DISCUSSIONS

Ciprofloxacin is a popular fluoroquinolone antibiotic that is primarily used to treat different bacterial infections. There have been worries about its potential neurobehavioral side effects, despite the fact that its usefulness in preventing infections is well-established. Numerous research have looked into the possible link between ciprofloxacin and neurobehavioral alterations. The effects on the central nervous system (CNS) are one area of interest. It is understood that ciprofloxacin can accumulate in the brain and can penetrate the blood-brain barrier. This has led to concerns about how it can impact neurobehavioral processes like cognition, emotion, and behaviour. There have been reports of neurobehavioral adverse effects connected to ciprofloxacin use in medical care. Confusion, agitation, hallucinations, depression, and even rare instances of spasms are possible side effects. It has been demonstrated to significantly affect the microbiota of the gastrointestinal tract. In order to sustain numerous elements of human health, such as metabolism, immunological response, and digestion, the gut microbiota is essential.

Few studies have examined the effect of repeated use and overuse of antibiotic Ciprofloxacin administration on the brain status and behavior at the adult age. One of the consequences of antibiotic treatment is an impaired microbiota (dysbacteriosis) in the gut leading to impairments in the immune system and overall metabolism. Even so, inadequate amount of information is available in the literature elucidating the interplay between antibiotic usage and brain biochemistry linking the use of antibiotics to stress and change in behavior.

Gut microbiota comprises a complex community of microbes, and is widely reported to affect multiple physiological processes of the host, including gut-brain communications, development and function of the brain, and behaviors. Antibiotics excessive usage induce

gut dysbiosis which leads to stress and neurobehavioral changes. Antibiotic treatment also alters the composition and magnitude of gut microbiota species and reduce species' diversity resulting in shifts in metabolism and mood physiology. Restoration of a healthy microbiome via the use of probiotics can reverse antibiotics-induced mental stress.

In this project, we have utilized BALB/c mice model to investigate the behavioral changes caused by disturbance of gut microbiome due to administration of antibiotic ciprofloxacin which is a commonly used antibiotic and investigated the efficacy of commercially available probiotics such as Enterogermina containing spores of ***Bacillus clausii*** in mitigating antibiotic-induced dysbiosis. The effectiveness of the probiotic bacterium Bacillus clausii in the management of diarrhoea has been thoroughly investigated. Frequent loose or watery stools are a common symptom of diarrhoea, which is frequently brought on by bacterial or viral infections, food intolerances, or modifications in the gut flora. The gut microbiota can also be repaired and stabilised by Bacillus clausii. The normal equilibrium of the gut microbiota is upset by diarrhoea, which results in an increase in potentially harmful bacteria and a decline in beneficial bacteria. The levels of beneficial bacteria can be elevated through the administration of Bacillus clausii, which can help improve gut health and shorten diarrheal episodes.

In this study, we examined the effect of Bacillus clausii in optimizing the neurobehavioral changes caused by the overuse of Ciprofloxacin. By this purpose, twenty-four 4-6 weeks old BALB/c mice were divided into 4 groups and three groups were exposed to a broad-spectrum antibiotic ciprofloxacin (120mg/kg) up to 3 weeks. Antibiotics were administered for three weeks, which was followed by one week of administration of probiotics without antibiotics in one group (treatment group1) and administration of probiotic simultaneously with antibiotic for three weeks in another group (treatment group 2) to avoid the side effects of antibiotics on gut microbiome and avoid stress. In positive control we only gave antibiotics to the mice for three weeks while in negative control no antibiotic was administered. After the antibiotic and probiotic treatment period, the mice were subjected to behavioral tests. The locomotor activity, anxiety, and depression-like behaviors of mice were identified along with the assessment of learning and memory performances. Later we performed microbial cultural test to estimate the microbial diversity and to confirm the presence of probiotic ***Bacillus clausii*** in the gut of mice.

Behavioral testing in animals is one of the most fundamental parts of assessing normal and diseased conditions of human. Abnormal social behavior, considered as an indication of neuropsychiatric disorders, could be assessed with behavioral tests. . Our results demonstrated some behavioral deviations in the antibiotic-treated mice.

The forced swim test is used to assess mice responsiveness to stress by observing their mobility during swimming. The decrease in immobility is an indicative of stress in mice. Maximum immobility time was observed in the antibiotic control group as compared to other groups. It indicates that Ciprofloxacin induced stress which impaired cognitive and memory functions in brain. Altered neuron transmission resulted in immobility in mice. Where as in experimental group 2, in which probiotics supplementation along with antibiotics were administered, showed highest mobility and mice responsiveness. This indicates that probiotic supplementation orevented gut dysbiosis leading to more activity and responsiveness in mice.

Tail suspension test shows the activity of mice when suspended in a downward position. The overall locomotor activity decreased by 3 to 4 mins in the mice treated with antibiotics only while the mice given probiotics showed much better results. In this test, experimental group 2 consistently showed beter results as compared to other groups, displaying positive effects of probiotic taken simultaneously.

In Marble burrowing test, we measure the stress and anxiety in mice by assessment of the innate ability of mice to bury the materials and interact with foreign objects. The test results indicated that the group of mice that were only exposed to antibiotic (antibiotic control group) showed the most anxious and depressed behavior as compared to treatment group 1 and treatment group 2 which were also treated with probiotics after being exposed to antibiotic.

In Elevated plus maze test, we estimated the anxiety of the mice by measuring the time spent in the closed arm vs open arms. The results indicated the mice which were given probiotics appeared to be less anxious than the antibiotic control group. The finding in these behavioral tests were consistent in reporting that being exposed to only antibiotics can change the behavior of the mice and lead to gut dysbiosis if they are not given probiotics. The results also revealed that the treatment group 2 exhibited better results than treatment group 1 henceforth, giving probiotics simultaneously with antibiotics produced better results than giving probiotics after treatment with antibiotics.

The largest microbial community occupies the gut in the human body: Its microbiome contains a wide diversity of microbial organisms. We performed cultural test on the fecal sample of mice to confirm the retention of ***Bacillus clausii*** in the gut of mice and to observe the impact of antibiotic administration on the microbial diversity. As

the culturing of fecal sample on general media (EBM) led to merging of colonies we progressed toward more specific media of tryptic soy agar (TSA) which led to more specific results. In order to confirm the nature of microbes and recognize ***Bacillus clausii*** in the media we performed gram staining as *Bacillus clausii* is a gram positive bacteria while the rest of the bacteria that grow on this specific media are mostly gram negative. In the results of gram staining of treatment group 2, showed that it retained purple color which is most probably the indication of presence of ***Bacillus clausii*** while other groups appeared mostly pink under digital microscope. As the retention of purple stain in treatment group 2, indicate the successful colonization of *Bacillus clausii*. The stress in these mice was reduced as evident in the results of behavioral tests.

So, the results from all tests performed were consistent with the hypothesis that probiotic ***Bacillus clausii*** which is used to reverse the diarrhea by inhibiting the growth of patogenic bacteria and proliferating the growth of benedficial bacteria; thus maintaining gut health, was successful in optimizing neurobehavioral changes caused to overuse of antibiotics. The study showed that probiotic supplementation taken along with antibiotic dosage prevents the gut dysbiosis most effectively. Hence, the prescription of probiotcs along with antibiotics could prevent the gut from side effects of antibiotics.

Chapter 6

FUTURE PROSPECTS

The probiotic *Bacillus clausii* was tested against modulating antibiotics-induced mental stress i.e., depression and anxiety like behaviors. However, its further functional properties can be studied in some other specific mental disorders such as Alzheimer's Disease or Dementia to explore the effect of probiotics on the brain. In this study, single probiotic was used in mitigating antibiotics-induced mental stress. In future, *Bacillus clausii* can be used in combination with other strains of probiotics to study their potential against different class of antibiotics. Furthermore, future studies can focus on longer duration of exposure to antibiotics and probiotics.

Chapter 7

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