الجمهورية الجزائرية الديمقراطية الشعبية
Democratic and Popular Republic of Algeria
وزارة التعليم العالي والبحث العلمي
Ministry of Higher Education and Scientific Research
جامعة مولاي الطاهر، سعيدة
MOULAY Tahar University, Saida

كلية العلوم
Faculty of Sciences
قسم البيولوجيا
Department of Biology

N° d'Ordre

Master's Thesis

In Biological Sciences

Specialty: Applied Microbiology

Theme

Study of the Combined Therapeutic Effect of Pomegranate Peel and Probiotic Strains Isolated from Camel Milk on Digestive Imbalance in Wistar Rats

Presented by:

- Ms : CHIBANI Ikram

Presented on : 25/09/2025

Before the jury composed of:

President	Ms Bounouala Fatima Zohra	MCB Univ AMAR Telidji Laghouat
Examiner	Mrs Belguacem Habiba	MCB Univ MOULAY tahar Saida
Reviewer	Mrs Amara Sabrina	MCB Univ MOULAY tahar Saida
Co-supervisor	Ms Tazi Lamia	MCB Univ MOULAY tahar Saida

Academic year 2024/2025

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Dedication

I dedicate this work, the culmination of perseverance and growth, to myself, Chibani Ikram, as a tribute to the strength, determination, and patience I've discovered along this academic journey.

With deep love and endless gratitude, I dedicate it to my beloved parents. Their unwavering support, sacrifices, and prayers have been the foundation of my success. May God protect them and reward them for their boundless love and faith in me.

To my dear family—especially my cousins—thank you for your constant encouragement, kind words, and comforting presence, which have uplifted me in moments of doubt.

And to my cherished friends *Maroua*, *Kheira*, *Rania*, *Hadjer*, and *Wissem*, your kindness, encouragement, and the laughter we've shared have made this path brighter and more meaningful. You've been my strength, and I'm endlessly thankful for you.

May this dedication reflect the depth of my appreciation for all those who have believed in me and walked beside me throughout this unforgettable chapter.

Chibani Ikram

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First and foremost, I humbly express my deepest gratitude to Almighty God for His boundless grace, enduring mercy, and for granting me the strength, resilience, and determination to bring this academic journey to completion.

I extend my sincere and profound thanks to my supervisor, Mrs. *Amara Sabrina*, Professor at the University of Dr *Moulay Taher* of Saida, for her unwavering support, insightful guidance, and invaluable advice throughout this project. Her mentorship was a true pillar of this work, and I sincerely pray that God blesses her children and fills her life with lasting happiness through them.

I am deeply thankful to Dr. *Hassani Zahira* for her constant encouragement and thoughtful feedback, which provided clarity and confidence during my research. I also extend heartfelt appreciation to Dr. *Tazi Lamia* for her kind support and attentive care—may God reward their efforts and grant them continued success in all their endeavors.

My sincere thanks go as well to the esteemed jury members for generously dedicating their time to evaluate this work. Their insightful remarks and constructive observations will undoubtedly enrich my understanding and future academic growth.

Lastly, I express my profound gratitude to all the educators whose dedication and expertise have shaped my academic path. Their commitment to knowledge and inspiration helped expand my intellectual horizons and nurtured my curiosity every step of the way.

Thank you.

Chibani Ikram

List of abbreviations

°C: Degrees Celsius

16S rDNA: 16S Ribosomal DNA

16S rRNA: 16S Ribosomal RNA

AAB: Acetic Acid Bacteria

Ace: Abundance-basedCoverageEstimator

AIEC: Adherent-Invasive *Escherichia coli*

C9O4: Probioticstrain code

$CaCO_3$: Calcium Carbonate

Caco2: Human epithelial colorectal adenocarcinomacell line

Caspase-3: Cysteine-asparticacidprotease 3

Catalase: Antioxidant enzyme

CBC: Complete Blood Count

CFU / CFU/g: Colony-Forming Unit / Colony Forming Units per gram

COX-2: Cyclooxygenase-2

CRP: C-ReactiveProtein

DALY LC8: Probioticstrain code

DNA: Deoxyribonucleic Acid

DNPH: Dinitrophenylhydrazine

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic Acid

F344: Fischer-344 (rat strain)

F. prausnitzii: *Faecalibacteriumprausnitzii*

F. sombrovi: *Faecalibacteriumsombrovi*

$FeCl_3$: Ferric Chloride

$FeSO_4$: Ferrous Sulfate

GALT: Gut-Associated Lymphoid Tissue

GI: Gastrointestinal

GF: Germ-Free

GM: Gut Microbiota

GN: Glucose Nutrient (culture medium)

GOSs: Galacto-Oligosaccharides

GRAN: Granulocytes

GRAS: GenerallyRecognized As Safe

GSK-3β: Glycogen Synthase Kinase 3 beta

H_2SO_4 / H2O2: Sulfuric Acid / HydrogenPeroxide

H9N2: Avian Influenza Virus subtype

HCl: Hydrochloric Acid

HCT116: Human colorectal carcinomacell line

HGM: Human Gut Microbiota

HPLC-PDA: High-Performance LiquidChromatographywith Photodiode Array

IBD: InflammatoryBowelDisease

IBS: Irritable Bowel Syndrome

IDD: IdiopathicDiarrhealDiseases

ICMR: Indian Council of MedicalResearch

IFN-γ: Interferon gamma

IL-1β: Interleukin 1 beta

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-17: Interleukin 17

IL-23: Interleukin 23

K. pneumoniae: *Klebsiella pneumonia*

LAB: Lactic Acid Bacteria

LB: Luria-Bertani

Lb. / L.: *Lactobacillus*

LG36: *Lactobacillus gasseri*strain

LMF: Low-MolecularFructan

LMPs: Low-MethoxylPectins

Lpb.: *Lactiplantibacillus*

Lp-1 / Lp62 / WCFS1 / PH07 / PMO 08: Probioticstrain codes

MRS: de Man, Rogosa and Sharpe (culture medium)

$Na_2\ HPO_4$: DisodiumHydrogen Phosphate

$NaH_2\ PO_4$: Sodium Dihydrogen Phosphate

NaOH: Sodium Hydroxide

NSC10: *Lactobacillus plantarum*strain NSC10

O157:H7: *Escherichia coli* O157:H7

OD_{600} nm: Optical Density at 600 nanometers

OTU: OperationalTaxonomic Unit

P38 MAPK: p38 Mitogen-ActivatedProtein Kinase

PAMPs: Pathogen-Associated Molecular Patterns

PCR: Polymerase Chain Reaction

PD: Pomegranate-derived

PI3K: Phosphoinositide 3-Kinase

PPE: Pomegranate Peel Extract

PP: Pomegranate Peel

Pico/lactocin: Bacteriocin type

Plantaris: Bacteriocin type

QPCR: Quantitative Polymerase Chain Reaction

QPS: Qualified Presumption of Safety

Rifampin: Rifampin (antibiotic used in susceptibility test)

ROS: Reactive Oxygen Species

rpm: Revolutions Per Minute

rDNA: Ribosomal DNA

rRNA: Ribosomal RNA

S. aureus: *Staphylococcus aureus*

SCFA / SCFAs: Short-Chain Fatty Acid(s)

SD: Sprague Dawley (rat strain)

SPF: Specific Pathogen-Free

SS: *Salmonella-Shigella* Medium

SW test: Shapiro–Wilk Test

THP-1: Human monocytic cell line

Th1: T-helper 1 cells

Th17: T-helper 17 cells

TLC: Thin-Layer Chromatography

TNF-α: Tumor Necrosis Factor alpha

V9 region: Variable Region 9 of the 16S rRNA gene

Vancomycin: Vancomycin (antibiotic used in susceptibility test)

VRBL: Violet Red Bile Lactose (culture medium)

WBC: White Blood Cell

w/v: Weight/Volume

v/v: Volume/Volume

µm: Micrometer

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Résumé

Cette étude a pour objectif d’évaluer l'effet préventif et thérapeutique combiné des écorces de grenade et de deux souches probiotiques de Lactobacillus plantarum (NSC10 et JUMII4), isolées du lait de chamelle, sur le déséquilibre digestif chez le rat Wistar. Contrairement aux approches classiques, le traitement a été administré avant l’induction du déséquilibre afin d’en mesurer le potentiel protecteur.

Les effets ont été évalués à travers le suivi du poids corporel, de la température, des dénombrements fécaux et de divers paramètres biochimiques. Les résultats montrent que les rats ayant reçu les probiotiques, prébiotiques ou leur combinaison ont présenté une meilleure stabilité physiologique et une nette amélioration de leur flore intestinale comparés aux témoins non traités. La souche JUMII4 s’est révélée particulièrement efficace.

Ces observations suggèrent un potentiel synergique entre les composés naturels des écorces de grenade et les probiotiques ciblés dans la prévention de la dysbiose microbienne.

Mots clés : écorces de grenade, Lactobacillus plantarum NSC10, JUMII4, probiotiques, prébiotiques, flore intestinale, rat Wistar, lait de chamelle, dysbiose, paramètres biochimiques, traitement préventif.

Abstract

This study aims to assess the preventive and therapeutic effects of pomegranate peels and two probiotic strains of Lactobacillus plantarum (NSC10 and JUMII4), isolated from camel milk, on intestinal imbalance in Wistar rats. Uniquely, the treatment was administered prior to the induction of digestive disturbance in order to evaluate its protective potential.

The effects were measured through changes in body weight, temperature, fecal bacterial counts, and several biochemical parameters. The results showed that rats receiving prebiotics, probiotics, or their combination exhibited greater physiological stability and a significant improvement in gut flora compared to untreated controls. The JUMII4 strain stood out for its efficacy.

These findings highlight the synergistic potential of natural compounds from pomegranate peels and targeted probiotics in supporting digestive health and preventing microbial dysbiosis.

Keywords: pomegranate peels, Lactobacillus plantarum NSC10, JUMII4, probiotics, prebiotics, gut microbiota, Wistar rats, camel milk, dysbiosis, biochemical parameters, preventive treatment.

ملخص

تهدف هذه الدراسة إلى تقييم التأثير الوقائي والعلاجي المشترك لقشور الرمان وسلالتين من البروبيوتيك Lactobacillus plantarum (NSC10 et JUMII4) المعزولتين من حليب الإبل، على الاضطراب الهضمي لدى جرذان الويستار. بخلاف الطرق التقليدية، تم إعطاء العلاج قبل التسبب في الاختلال الهضمي لاختبار فعاليته الوقائية.

تم تقييم التأثيرات من خلال مراقبة الوزن، ودرجة الحرارة، وعدد البكتيريا في البراز، وتحليل بعض المؤشرات البيوكيميائية. أظهرت النتائج أن الجرذان التي تلقت المعالجة بالبروبيوتيك، أو بالبريبايوتيك، أو بكليهما، أظهرت استقرارًا فيزيولوجيًا أكبر وتحسنًا واضحًا في تركيب الفلورا المعوية مقارنة بالمجموعة غير المعالجة. وقد تميّزت السلالة JUMII4 بفعاليتها العالية.

تشير النتائج إلى وجود تأثير تآزري واعد بين المكونات الطبيعية في قشور الرمان والبروبيوتيكات المحددة في دعم التوازن الهضمي والوقاية من الاضطرابات الميكروبية.

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PART I. INTRODUCTION

Introduction

In recent decades, scientific advancements have deepened our understanding of the crucial role played by the gut microbiota in maintaining overall health. Composed of billions of microorganisms living symbiotically within the digestive tract, this ecosystem plays a central role in digestion, immunity, vitamin synthesis, and even in regulating brain function through the gut-brain axis. However, this delicate balance can be disrupted by various factors such as poor diet, stress, or the excessive use of antibiotics. While antibiotics are essential in treating bacterial infections, they can significantly alter the intestinal flora, leading to dysbiosis—an imbalance in the microbiota with both physiological and behavioral consequences.

In light of these concerns, many recent studies have turned toward natural alternatives, such as probiotics and plant-derived compounds, to restore gut microbiota and counteract digestive disturbances. Among these, pomegranate peels, rich in polyphenols and antioxidants, have drawn growing interest for their anti-inflammatory and antimicrobial potential. Likewise, specific strains of Lactobacillus plantarum, especially those isolated from camel milk, have shown strong resilience to gastrointestinal conditions and demonstrated beneficial effects on gut flora.

This study falls within that framework and aims to evaluate the combined preventive and therapeutic effect of pomegranate peels and a probiotic strain of Lactobacillus plantarum on experimentally induced digestive imbalance in Wistar rats. Unlike conventional curative approaches, our protocol involved administering treatment before the onset of the imbalance in order to assess its protective properties.

Our work is organized in two main parts:

· A bibliographic section, introducing key concepts about gut microbiota, probiotics, pomegranate peels, and their possible role within the gut-brain axis;

· An experimental section, consisting of:

o In vitro tests, focusing on the purification of the selected strains, their inhibitory capacities, and antibiotic resistance;

o In vivo tests, conducted on Wistar rats, to evaluate physiological (weight, temperature), biochemical, and microbiological (fecal count) parameters and the treatment's impact on digestive balance.

Through this study, we aim to contribute to a deeper understanding of how natural interventions can modulate the gut microbiota and offer promising strategies to preserve digestive health.

PART II. CHAPTER ONE

II.1. SECTION ONE : The Gut Microbiota

II.1.1. Introduction to Gut Microbiota

The gut microbiota is an increasingly popular area of research due to the promotion of human health by diet, prebiotics, and probiotics and the discovery of the importance of gut microbiota for various diseases. Commensal microbiota has an important role in the maintenance of host metabolism, immunity, and gut nerves, and might offer adaptation to diet and host phylogeny. Within mammalian taxa, gut microbiota composition is shaped directly by host evolutionary history, phylogeny, and ecology. For instance, human gut microbiota is strongly affected by the use of antibiotics and diet. Insufficient knowledge on the rat microbiota, which is frequently used in experiments to study human health and diseases, might lead to unintentional introduction of unknown confounding factors in experiments, which might mask or mimic responses to tested factors. As similar to humans, there is increasing interest in the application of natural prebiotics to alleviate disease in Wistar rats (Flemer et al., 2017).

The introduction of Wistar rats to a germ-free environment induced formation of a microbiota comprised of approximately 65 species, which was less diverse than the healthy gut microbiota of laboratory rats. As laboratory rats have been adapted to their diet, it is not surprising that the gut microbiota of Wistar rats raised under the same low-bandwidth environment shares more features with that of laboratory rats than humans. More details of Wistar rats and of the timeline of pilot studies are in the Methods section. The aim was to address to which extent biological and clinical findings in Wistar rats can be extrapolated from human gut microbiota. The analysis was based on microbiota present in feces uploaded in the public database for Group Y3, which contains two cohabitated Wistar rats fed the same chow. The possibility that both Wistar rats independently acquired the rare F. prausnitzii and F. sombrovi. This is of concern as the metabolic activity of a few species might sum misleadingly to elucidated features of culture-rich microbiota.

II.1.1.1. Definition and Importance

Specific bacteria begin to colonize the digestive tracts of rats after birth, when the microbiota is still quite sparse. Over time, this community matures and stabilizes. However, the composition of the gut microbiota in Wistar rats varies between sources, which warrants the characterization of these communities by DNA sequence analysis before experiments are conducted and confounding factors are introduced (Flemer et al.,

2017). Control over various factors has made the Wistar rat a suitable laboratory animal. Nevertheless, the long temporal and environmental history unrelated to the studied diet/drugs make such models somewhat less restricted than human subjects. Wistar rats (HsdWistar) were bred without selection from rats imported from the Wistar Institute in Pennsylvania in the late 20th century to Germany. Housing, breeding, and handling procedures at the feed seller spanned 2-3 months prior to arrival for the gastrointestinal physiology study. To minimize environmental factors, rats from the same breeding station should be systematically chosen, although it is acknowledged that there is no guarantee that all the environmental factors are the same or similar. Here, the analysis of the fecal microbiota of rats from the breeding source was carried out.

II.1.1.2. Historical Perspectives on Gut Microbiota Research

Being naturally evolved, mammals from different lineages house a complex consortium of about 700 bacteria and archaea species on average. This gut microbiota performs several beneficial functions for host health, development, metabolism, and cognition in return for the nutrient-rich anaerobic environment offered (Flemer et al., 2017). Aberrant gut microbiota compositions (dysbiosis), together with alterations of the gut environment, undercells several gastrointestinal (GI) and systemic diseases. To better understand the composition and functions of healthy and dysbiotic gut microbiota in mammals, recent advances have been made in culture-independent sequencing and bioinformatics technologies. Notably, thousands of gut microbiota studies involving millions of samples spanning the whole lifespan catch the attention of the scientific, financial, and public communities.

The Wistar rat is one of the most widely used laboratory strains of rats. Clean, well-defined, and standard environments with low variations in microbial infections lead to reproducible results regarding health and behavior. Considered as basic research animals, Wistar rats serve extensively in biomedical and pharmaceutical studies. However, the gut microbiota of Wistar rats remains underexplored. Meanwhile, Wistar rats hold advantages over mice in oral ingestion, large sample size, surgical manipulation, and sequencing depth, providing a unique opportunity to connect widespread human gut microbiota studies for understanding host-microbe interactions, screening gut microbes as health-promoting probiotics, and evaluating candidate probiotics in intervention studies. To this end, the composition and functions of gut microbiota in Wistar rats maintained in a clean environment at low microbial outgrowth have been overviewed.

Historically, the gut microbiota studies in Wistar rats incorporate two areas under the discipline of microbiology from late 19th to early 20th centuries. Firstly, under non-sophisticated microscopic observation, the gut microbiota was depicted as diverse entities in terms of shape, color, and dimension. This exploratory phase witnessed the invention of gut microbiota sampling methods and preparation of gut bacteria with unshakable impacts on conventional bacteriology. Secondly, genetic phenotyping was initiated based on the rationale that different germfree (GF) conditions would lead to various gut microbiota compositions. Recently, bioinformatics-based data-independent approaches refined phylogenic ancestry from GI dissection to treatment selection. However, using only one commercially available sequencing platform may overlook low abundant microbiota without appropriate computational adjustment.

II.1.2. Human Gut Microbiota

The stomach microbiota of people and other higher non-human mammals (including rats, chimpanzees) is presently important to physiology and health: it plays a role in digestion and metabolism; the transient entry of opportunistic pathogens in the common gut lumen can lead to different pathologies, generally involving inflammation, which is mediated via the interactions of their products with the human immune system. These gut organisms have been shown beneficial for health. Using animals in gastric microbiota studies, however, mostly involved other small mammals such as the conventional (inbred laboratory) mouse. Not only did the two largest phylogenetic branches of bacteria, forming the two domains, evolve independently for billions of years due to their extremity (ether-linked) cryptopleuricity, the emergence of the much smaller (less than 6000 species) Belly domain of archaea-the methanogens and relatives-also took 3.5 billion years. The research team of experts is responsible for the design of a systematic program for addressing how comparable the mammalian and rat stomach microbiotas are. The second major goal is to extend insights about the oral cavity bacteria of rats.

In the context of cohort studies in human gut microbiota, the standard of communication, documentation and organization has been highly influenced by projects like the Human Microbiome Project or MetaHIT, but none of that happens for other mammalian species, and including a Wistar rat group in such studies would mean they would be on their own. Thus, practical matters come to play a major role, which nevertheless have scientific and methodological considerations. Rats are cheaper to procure, house and maintain than mice. There are no challenges of generation time or

speed of access to germ-free facilities, as rats are either conventionally bred or readily available as such from several commercial sources (Flemer et al., 2017). The commercial vendors of rats, as with similar mice vendors, retain the proprietary rights to detailed pedigree information. All else equal, animals bred from animals with known pedigree are assumed to be preferential, and that is the situation with humanized mice in gnotobiology.

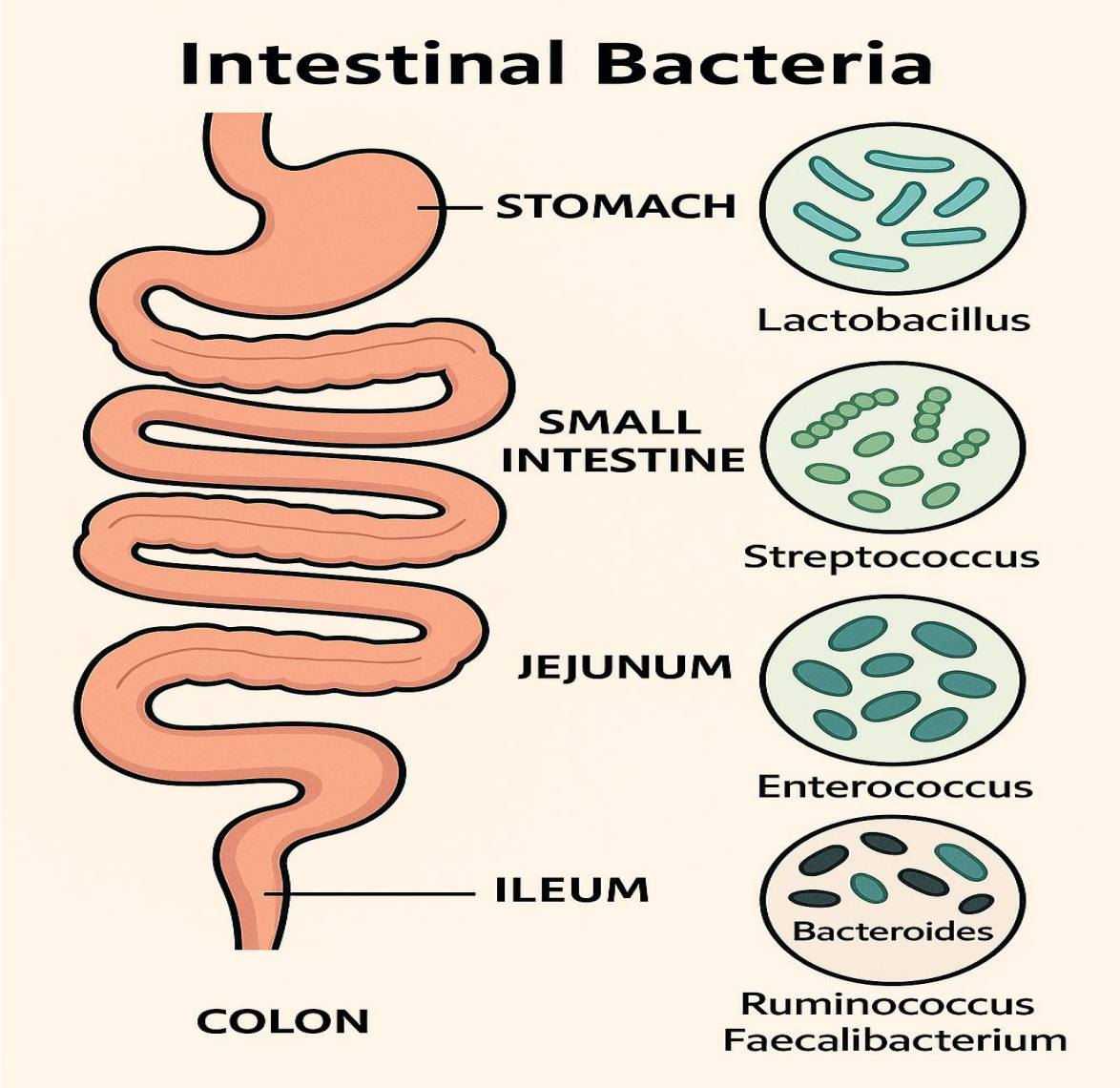


Figure 1: Schematic Representation of Human Gut Microbiota Distribution Along the Gastrointestinal Tract.

II.1.2.1. Composition of Gut Microbiota in Wistar Rats

Previous studies have characterized the human gut microbiota in healthy individuals, providing a comparative baseline for animal models. In this project, the gut microbiota of Wistar rats was analyzed to assess its response to combined treatment with pomegranate peel extract and a probiotic strain. Microbial composition was evaluated using 16S rDNA sequencing, allowing identification of key bacterial groups involved in digestive balance. Diversity analyses (Alpha and Beta) were conducted to observe shifts in microbial populations across treatment groups. Additionally, metabolic activity was assessed using bioluminescent assays, offering insight into the functional impact of the interventions. These findings contribute to understanding how natural compounds may influence gut health through microbiota modulation.

II.1.2.2. Factors Influencing Gut Microbiota

The composition of gut microbiota is shaped by multiple factors, including diet, lifestyle, medications, host genetics, and environmental exposure. While human microbiome research has advanced significantly, rodent models—particularly mice—have dominated laboratory studies, creating a gap in comparative data. Recent research highlights the value of Wistar rats as alternative models due to their physiological and microbial similarities to humans. Advances in molecular techniques, such as QPCR and 16S rDNA sequencing, have enabled more precise identification of bacterial communities in rat gut ecosystems. These tools support the exploration of therapeutic interventions, including plant-based compounds and probiotics, by revealing shifts in microbial diversity and metabolic activity. Understanding these influences is essential for evaluating the potential of natural treatments to restore digestive balance in experimental models.

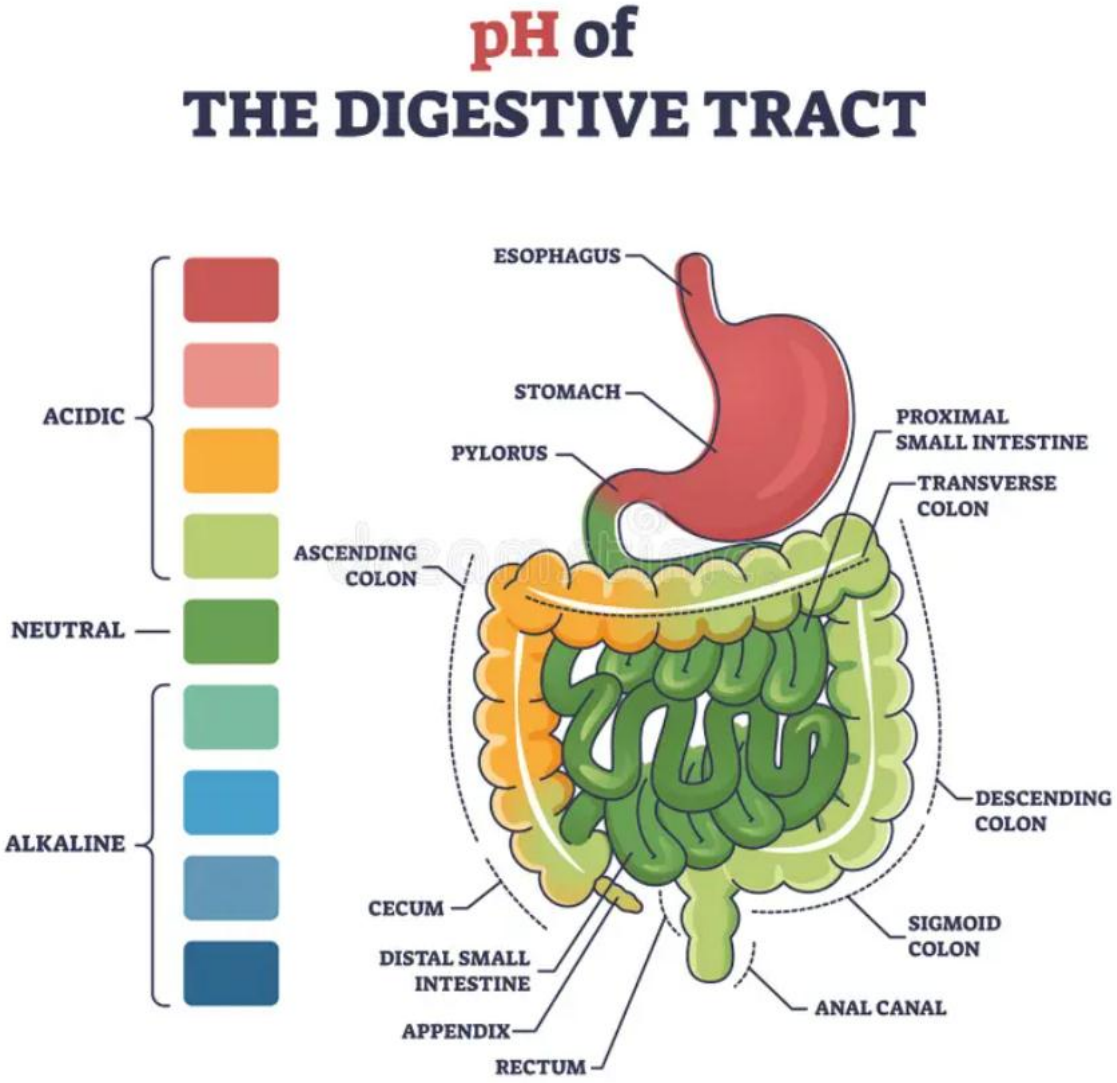


Figure 2: pH Gradient Along the Human Digestive Tract

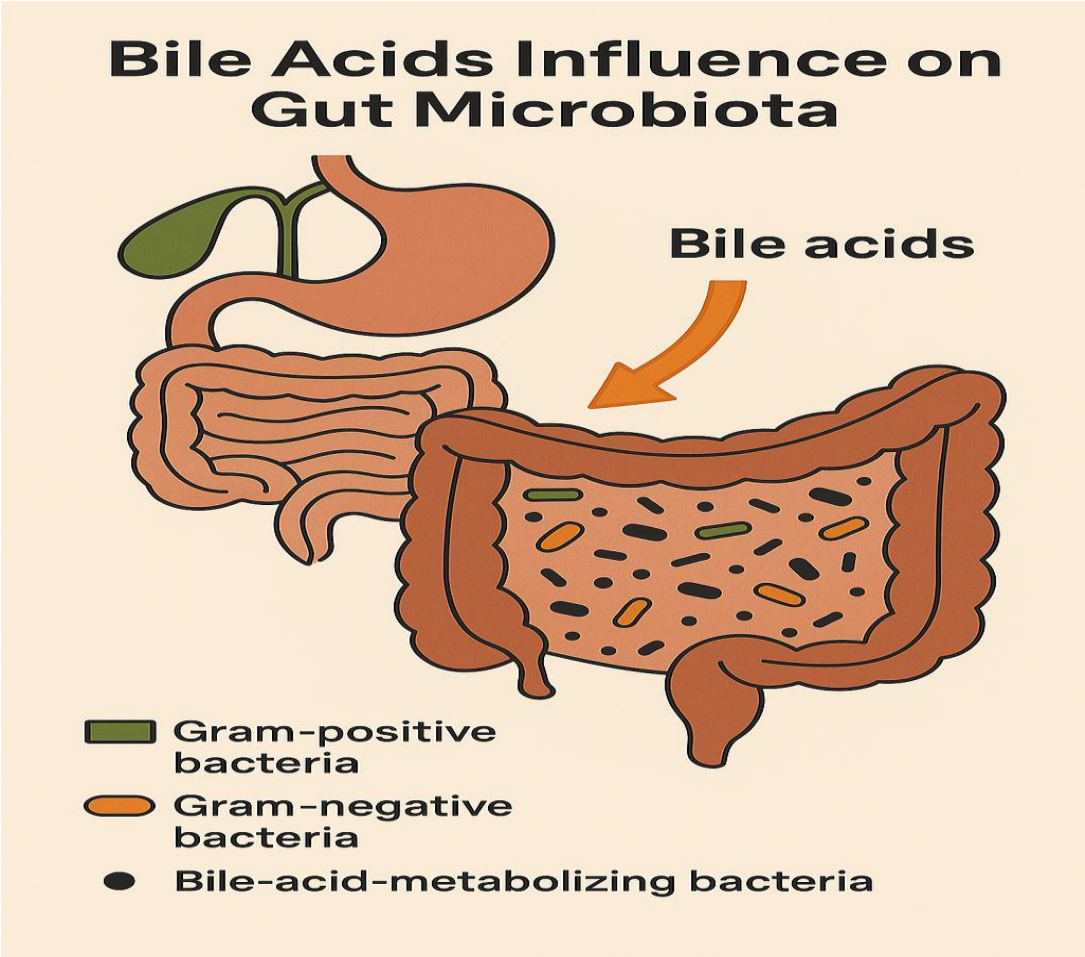


Figure 3: Modulatory role of bile salts on gut microbiota composition and metabolism.

This schematic highlights the antimicrobial effects of primary bile acids in the small intestine and the microbial transformations (e.g., deconjugation and dehydroxylation) occurring in the colon. It illustrates how bile salts shape microbial diversity, favoring bile-resistant species such as Bacteroides and Bilophila, while suppressing sensitive taxa.

II.1.2.3. Health Implications of Gut Microbiota

The study of the gut microbiome composition and function has garnered increasing interest as one of the most important human investigation areas of the decade and immediately attracts the attention of scientists in various fields. New scientific insights into the gut microbiome, uncovering a better intestinal microbial ecology and its dysfunctions, have been linked to several diseases, such as inflammatory bowel disease, autism, and colon cancer. Unlike classical model organisms, such as mice and flies, laboratory rats remain surprisingly underexplored, although rats have a long-standing history of being intensively studied due to their usefulness for highlights in translational medicine.

Therefore, understanding the rodent model in microbiome research is the first task. The availability of novel methods for fecal specimen preservation has greatly expanded the options for noninvasive collection, preserving the longitudinal nature of the experiments and ensuring that methodology remained unchanged.

In view of the efforts in studying human microbiome research, the gut microbiome's properties in Wistar rats were examined by analyzing 16S amplicon sequencing data. The utmost goal of this work was not only to provide a gut microbiome baseline in Wistar rats generated by human effort but also to demonstrate the feasibility of deducing insights from humans to Wistar rats. The gut microbiota dynamics in Wistar rats across their lifespan were carefully characterized. Because the gut microbiota is thought to resemble those of humans more closely compared with those of standard mice, the basic properties of the gut microbiota in Wistar rats were determined (Flemer et al., 2017).

II.1.2.4. Methods for Studying Human Gut Microbiota

The human gut microbiota is an important factor in health and disease, making it a target for microbiota-based therapeutic approaches. Several methods have been developed to study gut microbiota composition, function, and connectivity to the host. Researchers have developed methods to stabilize, extract, preprocess, and evaluate gut microbial community DNA. Such methods have already been reported for humans and laboratory animals, but not for Wistar rats. The first aim of the study is to use a simplified wet lab and bioinformatics workflow to extract gut microbiota DNA from Wistar rats. It has been shown that gut microbial DNA can be extracted using disposable sterile spatulas, and no PCR amplification is required. Accordingly, it is possible to study rat gut microbiota using commercial human-naïve kits.

A growing body of evidence demonstrates a link between gut microbiota and chronic neuroinflammatory/degenerative processes related to experimental autoimmune encephalomyelitis and multiple sclerosis. Tracing bacteria and their metabolites along the gut-brain axis in gnotobiotic animal models harbors great potential to provide insight into the functional connectivity of the gut microbiota and its host. Although several DNA extraction procedures have been developed to study microbial populations in human stool samples, no DNA extraction methods have yet been validated in the context of studying community composition and function in F344, Fischer-344, and Lewis rat strains.

Wistar rats are often used as a laboratory model to study neuroinflammatory and neurodegenerative processes. The DNA-stabilizing effects of several preservatives from different manufacturers and the feasibility of their application to the collection and storage of stool samples in rodent microbiota studies were tested. The effect of sample storage for several days at 8 °C or freezing, using a commercial extraction kit was validated for older dry powders stored at room temperature. The composition and diversity of the DNA from rat stool samples before and after preservation were evaluated using 16S rRNA gene sequencing, and 12 phylogenetic groups based on custom-built databases of more targeted taxonomic resolution were investigated.

II.1.3. Wistar Rats as a Model Organism

Rodents have long been established as model organisms for a variety of research fields, and rats, in particular, share with humans many physiological, pharmacological, and metabolic traits. The latter, however, appear to be underestimated in the context of gut microbiota research. Studies in Wistar rats (Rattus norvegicus) can now be prospective in monitoring variations in their fecal microbiota in response to acute and chronic dietary modifications or to pharmaceuticals, and piloting the experiment in rats before possible human studies. The inclusion of human cohorts with the same predefined criteria assessed in the rat cohort provides an opportunity to analyze how gut microbiota variations recapitulated in Wistar rats correspond to those in humans.

This research investigates whether the gut microbiota of Wistar rats (Rattus norvegicus) shares similar features with that of humans in terms of composition and variations in response to environmental stimuli, with an emphasis on dietary modifications. Rats were fed identical diets composed notably of a high dose of emulsified fish oil as omega-3 polyunsaturated fatty acids or no fish oil (control diet). Rats were sampled at various times after starting the diet. So-called donor subjects were all adult and healthy, with well-defined inclusion/exclusion criteria. Experience in a diet-induced shift in gut microbiota was essential prior to piloting experiments in rats.

Wistar rats have a different but complementary biodiversity regarding gut microbiota relative to humans. Such an experimental setting provides a controlled environment to facilitate subsequent, prospective, and randomized studies in Wistar rats. The potential repetition of experiments with similar statistical power in different laboratory environments and translation to human cohorts recognized as representative of the

corresponding study subjects bolsters the versatility of this design for other laboratory rodents. Eco-evolutionary studies of other mammalian orders with a priority towards non-human primates species may benefit from the current approach. This rhombus-shaped model could also extend to non-mammalian poikilothermic model organisms (Flemer et al., 2017)

Figure 4: Wistar Rats as Translational Models for Human Gut Microbiota Studies

II.1.3.1. Overview of Wistar Rats

Wistar rats (Rattus norvegicus) originated from a small group of albino laboratory rats bred at the Wistar Institute. They are healthy, non-inbred laboratory rats that are commonly used for pharmacological, toxicological and metabolic studies. Wistar rats are considered a standard strain in laboratory animal science, along with SD and Fisher 344 rats (Flemer et al., 2017). Their use in research has become indispensable partly due to their physiological, pathophysiological and histopathological similarities to humans. The anatomical, physiological and biological affinity to humans makes the experimental settings similar to human conditions when using Wistar rats in research. Owing to these properties, they have been widely used in biomedical and cognitive neurological studies. Because they are hardy rodents with relatively easy maintenance and breeding, they have been widely used for laboratory animal bioassays, in basic research and educational institutions, and for behavioural observations.

The Wistar rats used in the current study were bred, housed and examined at National Institute of Physiology, ICMR, India. The rats were six-week-old on purchase.

After acclimatization for a week, the rats were exposed to different protocols for behavioural tests that were conducted for about seven weeks. During the study the rats were housed in a temperature- and humidity-controlled room on a 12 h day-night cycle with free access to food and water. Group-housing type was used for housing along with proper nesting material. Temperature and humidity were monitored on a daily basis. In compliance with the ethical guidelines of the Institute Animal Ethics Committee, the number of rats recommended for each protocol was used. At the end of behavioral testing, the rats were deeply anaesthetized with isoflurane vapour and euthanized by decapitation, and tissues were harvested for analysis. All anaesthesia and environmental exposures were approved by the Institute Animal Ethics Committee. All efforts were made for minimizing the pain and discomfort during experiments.

II.1.3.2. Significance of Wistar Rats in Research

Revised research use Wistar rats (Rattus norvegicus) to detect novel bacteria in the human gut microbiome. Although rodents are the main model for gut microbiome studies, prior literature on their gut microbiome is limited. The research will go ahead with Wistar rats due to their characterisation as an outbred strain, wider availability, relevance in translational research and most importantly, phylogenetic distance to humans, which has been shown to enable a broader discovery range of gut members compared with mice. Furthermore, availability of faecal samples from earlier studies of 12, 24 & 48 week old Wistar rats will facilitate inclusion of host-associated variables into analyses. Past studies characterising the gut microbiome of laboratory rats are largely cross-sectional (Flemer et al., 2017). As such, longitudinal variation across the lifespan is poorly understood. Wistar rats were chosen as they are well-fed laboratory rats on a chow diet, which has been shown in mice to have less variability in gut microbiota than genetically modified diets, and restrictions preventing diet changes are placed on animal models in mortality studies.

II.1.3.3. Comparative Anatomy and Physiology

Wistar rats (Rattus norvegicus) are the most commonly used rodent model to mimic human gut microbiota. However, extrapolation of gut microbiota insights from humans to Wistar rats should be conducted with care. While fecal microbiota diversity, co-occurrence and stability increase to a greater extent in germ-free rats than in germ-free mice, distinct gut conditions in mice and rats such as diet preference, metabolic interaction and immune system, may also affect microbial composition and colonic structure. And, differences in

anatomy and physiology between human and rat, though smaller than those between human and mouse, must also be addressed.

Wistar rats are similar to humans in dental arcade and salivary secretion, while those in mice are highly divergent. In the four-chambered stomach of rats, a well-defined glandular and non-glandular portion with very different structure and secretion-type exists, whereas in mice, a much simpler stomach anatomy and structure is present with only luminal secretions. The sonde of duodenum is longer in rats and mice than in humans, but the relative length of pendulous and ampulated portions is similar. In total, the length of intestine is 3.7, 14.9 and 1.4m in rats, mice and humans, respectively. A conspicuous sacculus rotundus enters the cecum in rats but not in mice. Overall, rodents possess a more complex post-gastric digestive tract than other mammalian species, and much more so than did the ancestor of placentals. As a consequence, the colonic surface area of rats is significantly larger than that of mice.

Wistar rats are also different from humans in their daily feeding habit. Day-hunters such as humans usually exert energy as physical activities and exhibit a peck feeding pattern, while night-hunter rats obtain diet in bulk. Such a feeding habit difference is expected to affect post-prandial gut environments and the circadian rhythms of gut microbiota. To mimic the daily feeding habit of humans for studies on colonic fermentation, a separately constructed feeding matrix should be took into consideration. A rat-housing condition under temperature of 22±5 Re and humidity of 65%±5 and a well-ventilated specific pathogen-free environment is widely recommended in microbial studies, but amplification of unwanted bacteria is still unavoidable in a certain extent, especially within the cages and pens not cleaned regularly and during high temperature and humidity

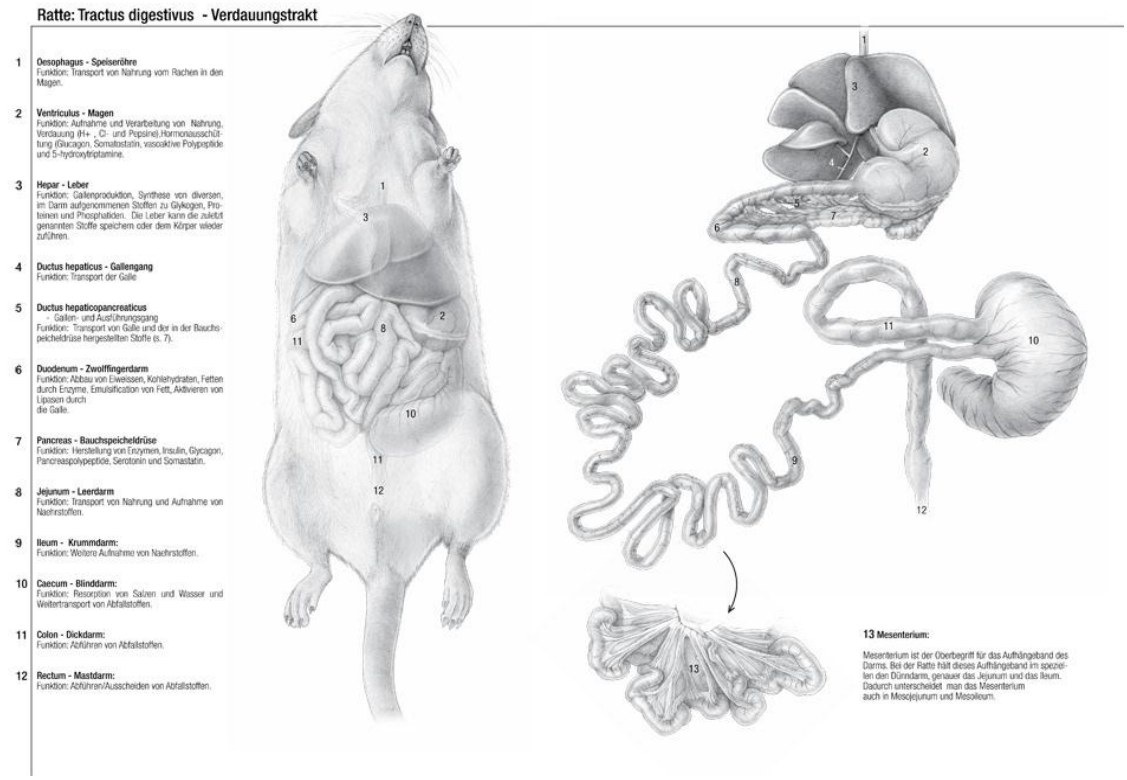


Figure 5: Anatomical Overview of the Digestive System in Wistar Rats

II.1.4. Wistar Rat Gut Microbiota

While previous studies have explored the gut microbiota of Wistar rats in comparison to humans and other laboratory animals, this project builds on that foundation by examining how targeted natural interventions can influence microbial composition. Using 16S rRNA gene sequencing, the current study investigates changes in gut diversity following treatment with pomegranate peel extract and a probiotic strain. Rather than reiterating existing comparisons, the focus here is on assessing therapeutic potential through microbial profiling and metabolic activity. This approach contributes to a growing body of research that positions Wistar rats as valuable models for studying digestive health and microbiota modulation.

II.1.4.1. Composition of Wistar Rat Gut Microbiota

A recent study characterizing the fecal microbiota composition in Wistar rats using 16S rRNA sequencing shows a relative abundance of Firmicutes (60.1%) and Bacteroidetes (23.4%), constituting 83.5% of the total community (Flemer et al., 2017). Similar to humans, the relatively abundant genera in Wistar rats were Bacteroides and

Lactobacillus. Notably, the gut microbiota diversity was lower in Wistar rats than in humans, analysis is focused on the 9270 common operational Taxonomic Units (96% similarity) in both species. The Firmicutes/Bacteroidetes ratio was higher in Wistar rats in comparison to humans. The composition of gut microbiota in Wistar rats is similar to that of humans. The relative abundance of Firmicutes was similar between humans and Wistar rats (equal to 60.1%), with the members from Clostridia class prevailing, and abundant genera being Lactobacillus, Faecalibacterium, and Ruminococcus. However, in Wistar rats, the proportion of Labaminas genus was much higher. The relative abundance of Bacteroidetes in Wistar rats was close to that in humans (23.4%). The composition of the gut microbiota composition was evenly variable in Wistar rats and humans. Genus Bacteroides is abundant in both species. Consistent with previous studies, several genera such as Bilophila, Dorea, and Eubacterium in Wistar rats are also relatively abundant in humans. A comparison of the composition in humans and Wistar rats indicated substantial degree of conservation. Such conservation suggested widespread gut microbiota-brain-gut axis mechanisms. With protein-free diets, the gut microbiota-related metabolic pathway in Wistar rats shifted from B vitamin synthesis towards the destruction of B vitamin. The consumption of B vitamin trace elements in rats can increase the activity of gut microbiota-related B vitamin metabolism, which agrees with the observed high abundance of gut microbiota pathway with metabolic B vitamin in Wistar rats. However, compared to humans, Wistar rats on commercial diet sue a weaker dependence on gut microbiota-generated B vitamins for gut bacteria.

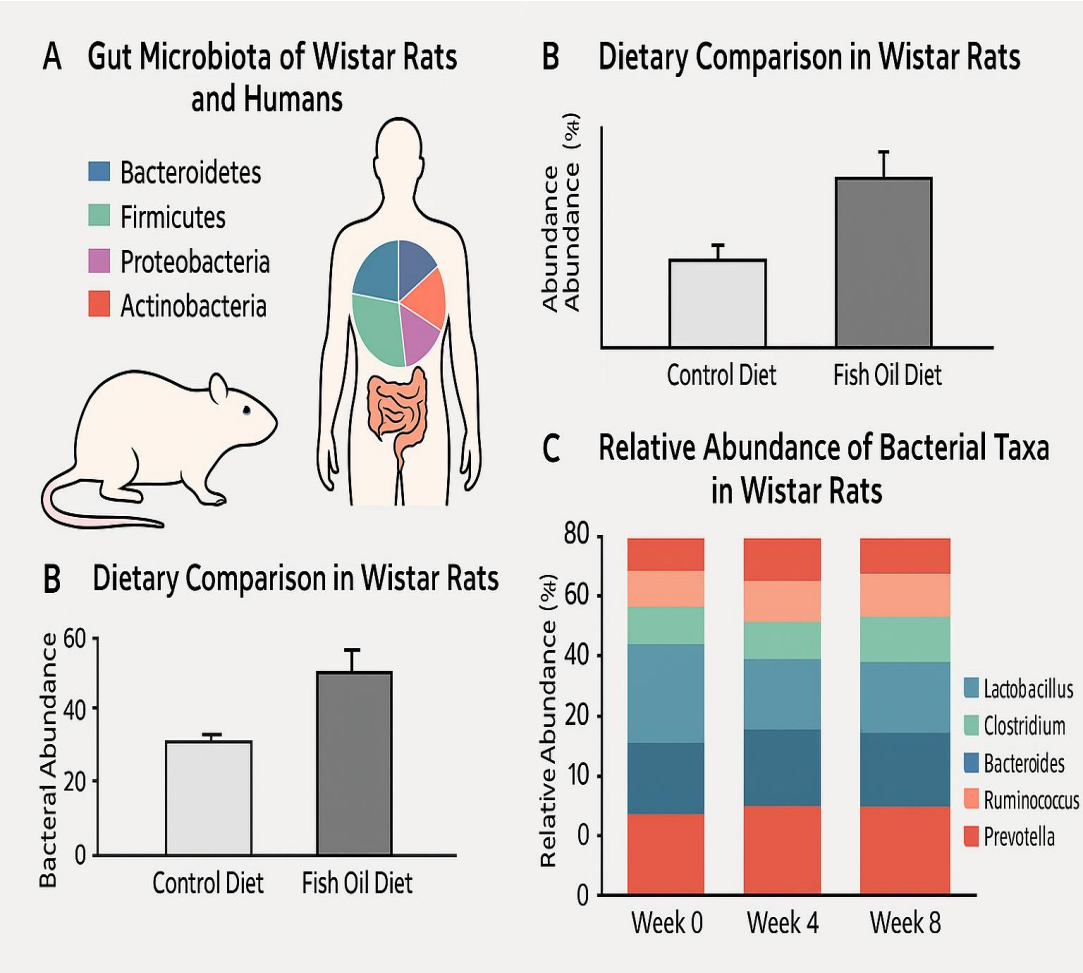


Figure 6: Gut Microbiota Composition in Wistar Rats Following Dietary Modulation

II.1.4.2. Factors Influencing Wistar Rat Gut Microbiota

As a priori approach, an analysis of published studies is performed to explore which factors might influence gut microbiota in Wistar rats. Because the main focus is on factors significantly influencing gut microbiota in humans as well, only studies performed on Wistar, Sprague Dawley or Fischer rat strains are included (i.e. excluding studies on outbred strains, knockout strains and genetically modified strains).

(Flemer et al., 2017) examined changes in fecal microbiota composition throughout the lifespan of the Wistar rat, a commonly used laboratory rodent strain. Fecal sampling was performed from two weeks of age to adulthood in three sequencing runs, and fecal microbiota composition was analyzed in terms of α (within sample diversity) and β (between sample diversity) diversity. High-throughput sequencing indicated that robust changes occurred throughout the lifespan in terms of phylogenetic units. The investigation of changes in a cohort of seven healthy laboratory rats revealed a dynamic fecal microbiota

composition during the first 17 weeks of life, with α-diversity gradually increasing to adult levels. Wistar rat fecal microbiota in the adulthood appeared relatively stable, with no significant differences in β-diversity found between 17- and 80-week-old animals. Wistar rats harbor a rich and diverse fecal microbiota that covariate with historical gradients of Porphyromonadaceae and Bacteroidaceae.

Extraction-free amplification of the epithelial 16S rRNA gene and subsequent sequencing was applied to assess how microbiota change under a high-fat diet in 8-week-old Wistar rats. Excessive fat diets significantly changed the composition of gut microbiota, increased body weight, fat weight and serum glucose and lipids, and impaired glucose tolerance and insulin sensitivity in rats. The composition of gut microbiota in Wistar rats was altered by a high-fat diet, while the ratio of Bacteroidetes to Firmicutes was not significantly correlated with body weight gain. Firmicutes, Actinobacteria and Proteobacteria were highly correlated with body weight gain, fat weight and serum glucose and lipid parameters. Colyins and Muribaculaceae were identified as gut microbiota indicators of Wistar rats undergoing hyperlipogenesis.

II.1.4.3. Health Implications of Gut Microbiota in Wistar Rats

This article opens with a short summary of the gut microbiota in laboratory rodents. With different feeding regimes, cage types, and environmental factors considered in the comparisons, 53 studies on gut microbial diversity in mice and 18 in rats were reviewed.

Most studies investigated how a single factor perturbation affected gut microbiota composition. These factors included diet, antibiotic treatments, exercise activity, subgingival deposits, stroke, surgery, and tumor growth. Microbiota in control groups or age-matched groups were compared with these single-factor perturbation groups.

There are very few studies on rat gut microbiota. (Flemer et al., 2017) reviewed 18 studies to summarize the data on gut microbiota in rats. The gut microbiota of healthy laboratory rats changes throughout the lifespan. The diversity was highest at 27 days, dropped to a second low at 63 days, and finally slightly increased. No change occurred in overall composition in 3-6 week-old Wistar rats with a pre- or post-weaning diet change. The fecal microbiota of 8 and 56 weeks old rats were similar but significantly different from those at 1 month old.

Microbial communities in feces of rats from four different facilities were identified. The microbial community structures were different from one another, with facility A and C

communities being the most similar of all pairs compared. Their community were distinct from the microbial community of Fischer 344 rats imported from an external source. The gut microbiota structure of rats in facility B was drastically different from other facilities and was dominated by Proteobacteria, Firmicutes, Actinobacteria, and Cyanobacteria.

II.1.5.Comparative Analysis of Human and Wistar Rat Gut Microbiota

A comparative analysis between human gut microbiota and rat gut microbiota was performed using all available human data to further investigate the effectiveness of Wistar rats as surrogates for human gut microbiota studies. In terms of environmental factors, food is carefully controlled for lab-use Wistar rats on a normal chow diet and rats undergo specific pathogen free status. Human and rat faecal samples segregated well from each other with clear differences in gut microbiota composition. Nevertheless, three human faecal samples grouped closely with Wistar rat gut microbiota samples, with two human faecal samples quite similar to those of Wistar rats. Phylum-wise distribution of gut microbiota was significantly different between human and rat gut microbiota. The latter have a richer proportion of Firmicutes and a lower proportion of Bacteroidetes, Actinobacteria, and Proteobacteria as compared with the former. Wistar rats also show similar clusters with human gut microbiota at genus-level resolution.

Closer examination of individual genera (≥3% of total abundance) revealed that large differences in abundance existed in a number of genera, regardless of their proportions across samples with statistical significance. Analysis of non-dominant genera revealed similarities in gut microbiota composition between Wistar rats and humans. Unclassified bacteria in both groups were not abundant, and rat poop contained fewer unclassified genera although the statistical difference was not significant (Flemer et al., 2017). Children's gut microbiota showed high richness and diversity, with relatively more phyla, especially Actinobacteria. An interactive heat map was developed for genera found in two or more samples, allowing for a deeper investigation of the closer resemblance of Wistar rats with humans.

Out of 51 genera found in all groups, 74% (38 out of 51) of which were also found in Wistar rat and fecal samples from other populations. Out of 63 genera common in Wistar rats and humans, 84% (53 out of 63) were either identified in fecal samples from both groups, but only 48% (30 out of 63) of common genera were shared between Wistar rats

and human adult samples. In terms of genera specificity, there were more rat-specific genera than human-specific genera. Rodent-specific genera formed a cluster, indicating that rodent fecal samples are not suitable controls for human microbiota supplements.

II.1.5.1. Similarities Between Human and Wistar Rat Microbiota

Wistar rats are a well-established rodent species used as experimental animals for a wide range of biomedical research areas. Their use is ubiquitous in modeling human disease mechanisms of various origins, including disease caused by microbiota alterations. Despite species-specific sequencing efforts, there was no suitable Wistar rat gut microbiome sequencing data for the microbe-augmented modeling of human-associated diseases. With the recent sequencing of the murine gut microbiome and the availability of modern sequencing technologies, insights into the microbiota of Wistar rats enable the assessment of the suitability of this model species in microbiome studies.

Microbiota data from human cohorts were collected to assess the feasibility of studying human-associated microbiota in the Wistar rat model using modern deep sequencing technology. Samples with very few reads were discarded and those with >10,000 reads were retained. A total of 411 human microbiome samples were collected, 529 samples from another project, and 82 samples from preterm newborns. After the removal of the control sample and the reads with a Phred quality score lower than 20, 5688 reads remained for future analysis.

Recently, the gut microbiota variation throughout the lifespan of Wistar rats was investigated by sequencing the V9 region of the 16S rRNA gene with next-generation sequencing. It was observed that the microbiota developed throughout the lifespan of the rats, which is concordant with the patterns observed in Wistar rats. The convergence of the 23 Wistar rats was orchestrated by four predominant genera including Bacteroides, Lactobacillus, Escherichia and Coprococcus. It was also concluded that age and the animal facility environment are the main factors influencing gut microbiota of Wistar rats. The present analysis demonstrated that the gut microbiota of Wistar rats was altered after the relocation to the animal facility environment and adoption of a chow diet.

II.1.5.2. Differences Between Human and Wistar Rat Microbiota

The gut microbiota of male Wistar rats is expected to share similarities with that of laboratory-housed humans due to their comparable environment, reproductive history, and habitat homogenization. The gut microbiota of 6-week-old male Wistar rats housed in a

controlled environment and fed a standard diet was highly correlated with that of multi-ethnic, healthy, laboratory-housed human adults. A previous study showed significant differences in the gut microbiota of Wistar rats from different suppliers, indicating that Wistar rats had a greater variation in gut microbiota than humans, who are housed in the same cages (Flemer et al., 2017). It is speculated that different colonies of Wistar rats share a more homogenous microbiota composition due to laboratory standardization, environmental control, and restricted diet compared to humans. investigated the comparative analysis of gut microbiota from 10 healthy humans, 6 multi-ethnic, laboratory-housed adult humans, and 18 Harlan Wistar rats. The share of sequences in the Firmicutes phylum samples was generally lower in human samples than in rat samples. The second and third most abundant phyla in Wistar rat samples, Bacteroidetes and Proteobacteria, were also found in the human sample, although some human samples did not contain Proteobacteria but had Lentisphaerae, which were not found in rat samples. The analysis showed that rat sample microbiota is less diverse compared to human sample microbiota. This is consistent with the observation of lower Chao1, Ace, and Shannon index values in rat samples. The SW test statistic was significantly higher in Wistar rats than in human samples, indicating that the gut microbiota of Wistar rats is more homogenous compared to humans. A greater number of OTUs in humans compared to Wistar rats, consistent with the previously reported greater variability of human gut microbiota, supports the observation that Wistar rat microbiota is more homogeneous (2.17 OTUs per samples) than that of humans (252.35 OTUs per samples).

II.1.6. Understanding Intestinal Dysbiosis

II.1.6.1. Definition and Conceptual Framework

Intestinal dysbiosis refers to a disruption in the composition and functional characteristics of the gut microbiota, commonly observed in individuals affected by Inflammatory Bowel Disease (IBD), including Crohn's disease and ulcerative colitis. Despite its widespread use, the term "dysbiosis" remains loosely defined due to the complexity of microbial communities and limitations in current analytical tools, particularly those focused on alpha/beta diversity indices or isolated microbial taxa.

Rather than a uniform state, dysbiosis in IBD is highly heterogeneous across individuals. Microbial ecosystems in the human gut are composed of diverse niches, each influenced by specific host–microbe and microbe–microbe interactions. When contrasted

with microbiotic homeostasis, dysbiotic communities typically demonstrate a significant reduction in species richness—often up to a three- to five-fold decrease in fecal samples—accompanied by a loss of structural diversity. Pathobionts such as adherent-invasive Escherichia coli (AIEC), Campylobacter jejuni, and Salmonella enterica have been implicated in Crohn's disease, while the microbial profile in ulcerative colitis remains less defined, albeit similarly altered.

Dysbiosis should not be misconstrued as an infectious process. It results from multifactorial perturbations, including host immune modulation, environmental influences, and metabolic shifts, making its study and clinical interpretation particularly challenging. Nonetheless, its role as a potential initiator and sustainer of mucosal inflammation in IBD continues to be a major focus of translational microbiome research.

II.1.6.2. Classification and Pathophysiological Context

Several forms of dysbiosis have been described in both clinical and experimental studies, generally grouped into three main categories:

- **Microbial Imbalance**: A relative shift in the abundance of key taxa, including both depletion of beneficial microbes and expansion of opportunistic species.
- **Loss of Microbial Diversity**: Characterized by diminished taxonomic and functional heterogeneity within gut microbial communities.
- **Excessive Diversity Dysbiosis**: A paradoxical state where over-diversification may reflect disorganized microbial colonization and instability.

Recent meta-analyses in preclinical models have expanded this taxonomy to include "overly-altered," "partially-altered," and "focal-low-abundance" dysbiosis, emphasizing relative abundance shifts over absolute microbial counts. These alterations can influence host physiology through changes in glycan metabolism, short-chain fatty acid (SCFA) production, and immune signaling pathways, such as the activation of phosphoinositide 3-kinase (PI3K).

Functional gut disorders, including irritable bowel syndrome (IBS) and idiopathic diarrheal diseases (IDD), are frequently associated with distinct dysbiotic patterns. In particular, dysbiosis following antibiotic treatment has been implicated in the pathogenesis of post-infectious IBS. Animal models—especially murine and porcine—continue to provide valuable insights into microbial contributions to motility disorders, visceral hypersensitivity, and chronic inflammation.

In conclusion, while a singular, universally accepted definition of dysbiosis remains elusive, there is consensus that alterations in gut microbiota composition can compromise host–microbe symbiosis and promote disease progression. The development of robust biomarkers and standardized criteria for identifying dysbiosis is essential for translating microbiome science into clinical interventions

II.1.7. Contributing Factors to Intestinal Dysbiosis

Intestinal dysbiosis results from a combination of dietary, pharmacological, infectious, lifestyle, and environmental influences that alter the structure and function of the gut microbiota.

• Dietary Factors: High-fat, low-fiber, and protein-rich diets disrupt microbial balance and promote inflammation. Prebiotics and fecal microbiota transplantation have shown potential in restoring intestinal homeostasis, though more targeted clinical evidence is needed.

• Antibiotic Use: Antibiotics reduce microbial diversity, particularly in early life, increasing susceptibility to immune and metabolic disorders. While they are essential for treating infections like C. difficile, they often induce long-term dysbiosis, making restorative strategies critical.

• Infections: Pathogenic microbes such as Salmonella and Clostridium difficile disturb microbial communities and immune balance. Infection-driven dysbiosis can persist and predispose individuals to chronic inflammation and recurrent gastrointestinal issues.

• Lifestyle Factors: Stress, poor diet, and environmental toxins can compromise gut microbial stability. Dysbiosis induced by these factors contributes to immune dysfunction through pathways such as IL-23/IL-17 and affects intestinal barrier integrity.

• Environmental Influences: Early-life events—such as mode of delivery, breastfeeding, and antibiotic exposure—profoundly shape initial gut colonization. Vaginal birth and breastfeeding foster healthy microbiota, while Cesarean delivery and formula feeding may increase dysbiosis risk. These early exposures can influence long-term gut health and vulnerability to IBD.

II.1.8. Mechanisms of Intestinal Inflammation (Summary)

Intestinal inflammation involves immune imbalance, epithelial disruption, and excessive cytokine production, often triggered or exacerbated by gut microbiota dysbiosis.

II.1.8.1. Inflammatory Cascade and Gut–Brain Axis

Inflammation in the gut is marked by the release of pro-inflammatory mediators such as prostaglandins, COX-2, and nitric oxide, alongside infiltration of immune cells like neutrophils and macrophages. In severe cases, this can lead to tissue damage and intestinal ulceration. Under normal conditions, the gut maintains homeostasis through balanced host–microbiota interactions and contributes to neurological health via the gut–brain axis. Disruptions in microbiota composition are linked to neuroinflammatory conditions, including anxiety and cognitive dysfunction in IBD models.

II.1.8.2. Immune Response Activation

The gut's immune defense includes epithelial barriers, mucus layers, and gut-associated lymphoid tissue (GALT). A healthy gut fosters tolerance to commensal microbes, but dysbiosis can tip the balance toward excessive immune activation. This involves the recognition of microbial components (PAMPs) by intestinal dendritic cells and Toll-like receptors (TLRs), promoting the differentiation of Th1 and Th17 cells and initiating inflammation. The overactivation of innate and adaptive responses contributes to chronic immune-mediated diseases such as IBD.

II.1.8.3. Cytokine Release and Systemic Impact

Dysbiosis often increases gut permeability, allowing microbial antigens to stimulate immune cells and promote the release of inflammatory cytokines—such as IL-1β, IL-6, TNF-α, and IL-17. These signals contribute to local and systemic inflammation, especially in disorders like IBD, obesity, and diabetes. The imbalance between pro-inflammatory and regulatory cytokines disrupts mucosal immunity. Hence, cytokine-targeted therapies and microbiota-modulating treatments may offer strategies to control inflammation rooted in microbial imbalance

II.2. SECTION TWO:Probiotics and Prebiotics

II.2.1. Introduction to Probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host (Nouri et al., 2018). These beneficial microbes support digestive function and contribute to the balance of intestinal microbiota. Most probiotics belong to bacterial genera such as *Lactobacillus* and *Bifidobacterium*, and are known for their ability to ferment indigestible carbohydrates into short-chain fatty acids, which play a role in gut health and immune modulation (Teresa Rocchetti et al., 2021).

In recent years, non-dairy probiotic sources have gained attention due to increasing dietary restrictions and allergenic concerns associated with dairy products. In this study, probiotic strains were isolated from camel milk, a nutrient-rich, non-fermented product traditionally consumed in various regions. The selected strain was evaluated for its survival under simulated gastrointestinal conditions, as well as its potential to restore digestive balance when administered to Wistar rats. This approach aligns with the broader goal of identifying natural, functional probiotics suitable for therapeutic use.

II.2.2. History of Probiotics

In light of new investigations on how beneficial and safe microbes can reshape the gut microbiota, humans appear to have co-evolved with bacteria and other microbes. The potential of food-borne microbes can be addressed in the 'old friend hypothesis'. This hypothesis states that exposure to nonharmful or commensal microbes in foods may engage with the digestive tract's mucosal surfaces, fine-tuning the immune system and bolstering gut function. For decades, LAB have been extensively used in food fermentation due to their nonharmful nature. LAB on the market today include species from several genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Beneckea*. These synonyms and naming discrepancies sometimes confuse sources of LAB.

Lb. plantarum, a biocompatible *Lactobacillus* species, has long been recognized as one of the most versatile. It is a Gram-positive, non-motile, non-spore-forming, microaerophilic, mesophilic bacterium. Although *Lpb. plantarum* strains are typically isolated from fermented foods, they can be found in many niches, including the GI tract and other fermented foods. Like *L. pentosus*, *Lpb. plantarum* strains can produce a wide variety of food products, including wines, dough, pickles, meats, sausages, and raw vegetables. For decades, *Lpb. plantarum* strains have been used in the food industry as

starter cultures in producing cheeses, olives, and a wide variety of fermented foods and beverages (Teresa Rocchetti et al., 2021). Their main isolates, *Lpb. pentosus* and *Lpb. plantarum*, can be quantified in fossilized dairy products, such as dried cheese, in quantities of around 10^8 CFU/g.

Currently, *Lpb. plantarum* strains are also being tested for their health-promoting properties. An increasing number of high-quality meta-analyses and reviews have shown that the gut-associated *Lpb. plantarum* strains can exert health-promoting roles, as do well-recognized therapeutic probiotic strains like *Lactobacillus rhamnosus* GG and *Saccharomyces cerevisiae*. The recent census of public databases for commercially exploited *Lactobacillus* strains has indicated that the non-pathogenic and well-studied *Lpb. plantarum* is a missed opportunity in probiotic research.

II.2.3. Types of Probiotics

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host. They are primarily composed of lactic acid bacteria (LAB), such as *Lactobacillus* and *Bifidobacterium*, as well as beneficial yeast strains like *Saccharomyces*. LAB have been widely utilized in the fermentation of dairy and plant-based products due to their ability to survive gastrointestinal transit, adhere to the intestinal mucosa, and produce antimicrobial compounds. *Lactobacillus plantarum*, in particular, has shown versatile applications in both food biotechnology and health promotion. Similarly, *Bifidobacterium* species are predominant in the gut of breast-fed infants and are known to support immune function and protect against pathogens. In parallel, yeast probiotics have gained prominence in animal health and aquaculture, enhancing gut integrity and nutrient assimilation under stress conditions. Collectively, these microorganisms contribute significantly to maintaining gut microbial balance, preventing dysbiosis, and supporting host health across species.

II.2.4. Lactobacillus plantarum Overview

Lactobacillus plantarum is a rod-shaped Gram-positive bacterium of the genus *Lactobacillus* commonly found in fermented foods. These bacteria are included in the group called lactic acid bacteria (LAB), which are generally recognized as safe (GRAS) microorganisms (Teresa Rocchetti et al., 2021). *Lactobacillus plantarum* is a highly competitive LAB species that can be found in a great variety of ecosystems, including the gastrointestinal tracts (GITs) of mammals, fermented food products, plant material, soil,

and the rhizosphere of several plants. Fermented products with *L. plantarum* strains are a significant part of the human diet in several geographical areas since they produce various metabolites beneficial both to the fermentation process and to human health. The use of this LAB in the food industry as a starter culture has been widely applied for the production of many fermented foods such as vegetables, dairy products, and cereals.

Lactobacillus plantarum has been proven to be a potential probiotic strain isolated from a spontaneous fermentation of a natural cereal beverage (Park & Lim, 2015). This strain was able to survive the simulated gastrointestinal transit conditions and produced tight biofilm to a certain limit in abiotic and biotic environments. The study observed that this strain contributed to the decrease of the pathogenic strain *Escherichia coli* O157:H7 in turbid fermentation conditions. On the contrary, concerns about the safety of *L. plantarum* on the basis of its potential pathogenicity and ability to translocate remain. Therefore, this investigation was undertaken to characterize potentially the probiotic properties of *L. plantarum* CB18 in terms of their safety, technological properties, and in vitro probiotic performance. The potential probiotic strain was able to survive against 0.5–1.0% w/v bile salts at 37°C and resisted the low pH without the loss of viability. Most importantly, the strain did not carry any transferable or mobile antibiotic resistance genes. The inulin-type prebiotics (HPF and LMF) had a significant bifidogenic effect in an in vitro colonic fermentation by this strain. In conclusion, a meticulous evaluation of safety assessment showed that *L. plantarum* CB18 met the entero- and general probiotic criteria and deserves further in vivo studies.

II.2.5. Sources of Lactobacillus plantarum

Several *Lactobacillus plantarum* strains have been identified and obtained from various habitats including plants, pickles, olives, vegetables, fermented Chinese cabbage, food, Chinese fermented soybeans, sourdough, traditional fermented milks, fermented beverages, spontaneously fermented fish, the intestines of pigs, broilers, and ducks, the gut of healthy humans, and even faeces of worms. In China, fermented cabbage has been popular in the Northeast region for more than hundreds of years. Researchers isolated strains from fermented cabbage, one of which was identified as *Lactobacillus plantarum*—hereafter referred to as *L. plantarum*—which was initially named C-1 (Park & Lim, 2015). C and F are commonly used to refer to wet (brined) and dry for non-aqueous fermentation sauerkraut preparation. Based on the fermentation characteristics of C-1 and the dominant flavor compounds produced compared with the fermentation by the dominant strain *L.*

plantarum C-1, several commercial starter cultures with different composition have been developed. Even cultures claiming health benefits and allicin-reducing activities have been patented. Today, there is an increased interest in probiotic strains from food or environment.

Fermented fish products have been investigated for the presence of broad-spectrum anti-lactate-producing bacteria capable of producing bacteriocins to inhibit human pathogens. *Lactobacillus plantarum* E-31, isolated from fermented fish, demonstrated the ability to inhibit human pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. The bacteriocins produced by *L. plantarum* E-31 were identified as plantaricins that belonged to class IIa, possessing anti-listerial activity. A probiotic strain of *Lactobacillus plantarum* PMO 08, showing bio-protective effects against foot and mouth disease virus, was isolated from Bidhan 7 bovine and autologous species of bovine products. Probiotic strains, *Lactobacillus plantarum* PH07, isolated from the gut of healthy humans, inhibited pathogen growth against *Staphylococcus aureus* and *Enterobacter aerogenes*. Another *Lactobacillus plantarum* strain DALY LC8 with good probiotic properties was isolated from healthy human feces, which inhibited H9N2 avian influenza virus infection (Teresa Rocchetti et al., 2021).

II.2.5.1. Fermented Foods

Fermented foods have long been considered as an important part of traditional diets, and their health-promoting effects have been described by many studies. Since prehistoric times, fermented foods have been part of the human diet; during fermentation, lactic acid bacteria (LAB) develop in the food substrates. Fermented foods of various origins give rise to a wide variety of flavours, colours, and aromas, which remain a great attraction for consumers and offer great shelf stability.

During fermentation, lactic acid bacteria have the ability to impair the growth of other undesirable microorganisms in the food as well as in the gastrointestinal tract, which are beneficial for preservation and consumption. Fermented foods have long been considered as an important part of traditional diets, and their health-promoting effects have been described by many studies. The consumption trend towards natural products, particularly those that minimize the use of synthetic additives, has led to an increasing interest towards fermented foods. Traditional dairy products originated from cow, buffalo,

goat, and sheep milk exhibit a wide range of varieties based on their specific processing methods (Garcia-Gonzalez et al., 2021).

Lactiplantibacillus plantarum is one of the lactic acid bacteria that are extensively used worldwide in the fermentation of various foods. Notably, the strains of *Lpb. plantarum* cultured from fermented foods exhibit various functional and health-promoting properties such as antimicrobial activity, exopolysaccharide production, cholesterol-lowering effect, adhesion to epithelial cells, and/or inhibition of yeasts or fungi activity. On the other hand, many health-promoting effects of probiotics are likely to be strain- or species-specific, and probiotic strains are reviewed with their specific health benefits.

II.2.5.1. Probiotic Supplements

Based on the awareness of *Lactobacillus plantarum*, it seems to be a versatile species of lactobacilli present in diverse environments, including the gastrointestinal tract (GIT) of mammals. *L. plantarum* is employed as starter cultures in the food industry for its capacity to ferment and preserve food. This species is generally recognized as safe (GRAS) and has been included in the list of microorganisms with Qualified Presumption of Safety (QPS) (Teresa Rocchetti et al., 2021). Experimental evidence highlights the probiotic character of several *L. plantarum* strains, some of which are commercialized as health-promoting supplements. The status of probiotics indicates microorganisms that confer health benefits on the host upon ingestion. This depends on microbial properties such as the ability to survive the human GIT, colonize the intestinal mucosa, reinforce gut barrier function, preserve gut microbiota balance, stimulate immune responses, support digestive functions, and synthesize bioactive molecules.

The production of antimicrobials is associated with important probiotic properties of LAB. Antimicrobial compounds from lactobacilli can control the growth of potential pathogens, thus contributing to host health and food safety. The main antimicrobial chemicals produced by lactobacilli comprise ribosomally synthesized peptides, i.e. bacteriocins, and metabolic by-products such as hydrogen peroxide and lactic acid. The antimicrobials produced by probiotic lactobacilli may be suitable alternatives to conventional antibiotics and hold great potential in biomedical applications. We aim to survey recent papers bioprospecting the antimicrobial activity of characterized probiotic *L. plantarum*strains, focusing on the different chemical nature of antibacterial and antiviral agents produced, their action spectra, and the mechanisms underlying their bioactivity.

II.2.6. Health Benefits of Lactobacillus plantarum

Among all the species of probiotic strains used in foods and dietary supplements, *Lactobacillus plantarum* is the most frequently listed. Its health-promoting effects, beneficial functionality in fermented foods, and technological properties make it the most studied lactobacilli species in health, food, and nutrition research (Garcia-Gonzalez et al., 2021). In general, the mechanisms by which probiotic bacteria mediate their health benefits are: (i) modulation of commensal microbiota, (ii) exclusion or inhibition of pathogens, (iii) enhancement of the intestinal epithelial barrier by increasing mucin production and tight junctions formation, (iv) modulation of the immune system, and (v) production of bioactive molecules. These mechanisms may vary depending on the probiotic strains, their health effects, and the methods or assays used for the investigation. Health-promoting properties may be mediated alone or through interactions with other probiotics or components of the commensal microbiota. The modulation of the commensal microbiota and the inhibition of pathogens may lead to a reduction of microbes with putative effects on the disease improvement or prevention, and to an adherent microenvironment to the epithelium wall, which may also be mediated by bioactive compounds.

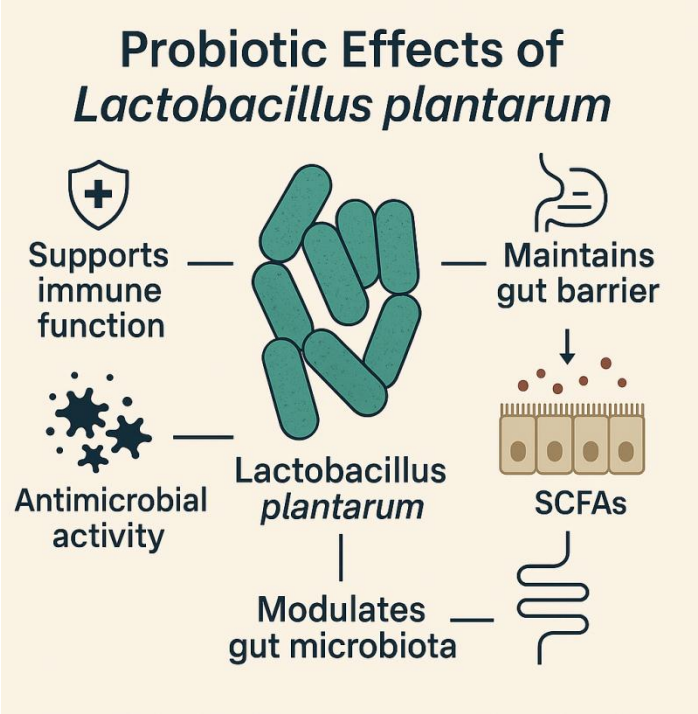


Figure 7: Mechanisms of Probiotic Action by Lactobacillus plantarumon Intestinal Health

II.2.6.1. Digestive Health

In recent years, there has been a growing interest in the use of probiotics, live microorganisms, which when administered in adequate amounts, confer a health benefit on the host. The human gut is an extraordinary complex ecosystem. To keep gut health, gut microbiota (GM)—an enormous collection of trillions of microorganisms, mainly bacteria, living in a symbiotic relationship with the host—need to be balanced. A dysbiosis has been mostly implicated in many disorders affecting health and well-being (e.g. stress, depression, psychological impairment). GM can vary geographically and through the human life span, nevertheless, a devisable core GM has been recognized, modulated by diet, lifestyle. Gut dysbiosis can be counteracted by food or substances containing probiotics: mainly bacteria but also yeasts. A proper diet, enriched in probiotics, will keep a balanced GM, modulating health positively (Garcia-Gonzalez et al., 2021). Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Probiotic organisms, alone or in combination, exert beneficial effects in sufficient numbers. They are taken by consumption of fermented foods, probiotic-thickened milks and nutritional supplements. Even if the criteria to recommend a bacterium as probiotic should be strictly followed, some microorganisms are commonly and improperly called probiotics, even when they have been poorly characterized.

Probiotic microorganisms are mostly Gram-positive bacteria with rod and cocci shape. *Lactobacillus* and *Bifidobacterium* are the genera most frequently used as probiotics in food supplementation and medical therapy. Probiotics also include a few strains of *Enterococcus*, *Streptococcus*, *Saccharomyces*. Since 2000, most strains of *Lactobacillus* and *Leuconostoc* genera have been reclassified as *Lactobacillaceae,* and their type species *L. acidophilus, L. casei*, *L. delbrueckii*, *L. fermentum, L. gasseri*, *L. johnsonii*, *L. lactis*, *L. leichmannii*, *L. plantarum*, *L. sakei*, *L. rhamnosus*, *L. reuteri*, *Leuconostocmesenteroides*, and *Oenococcusoeni* are used as probiotics widely.

II.2.6.2. Immune Support

The *L. plantarum* Lp62 probiotic strain increased the mixtures' pH during their storage and preservation in a dairy matrix. Higher acidity levels were observed for the inoculated mixtures, which also presented with better pH average values. However, the probiotic strain did not produce antimicrobial compoundages in fermented milk. It was concluded that both strains of *L. plantarum* used in this study positively influenced the pH,

titratable acidity, and organic acid production in fermented milk containing soy protein isolate or whey protein concentrate. The *L. plantarum* Lp62 exhibited an acidifying capability in soy-based yogurt and positively influenced the structure of the sensory profile of the product over the evaluated storage period. Furthermore, two strains of *L. plantarum* were able to produce, in fermented whey protein concentrate, higher values of total acidity and lactic acid than the control. It is suggested that probiotic *L. plantarum* Lp62 strain can be used as a new ingredient in soy yogurt manufacturing (Ferreira dos Santos et al., 2016).

Probiotic therapy has demonstrated efficacy in the treatment and prevention of diseases associated with an imbalance in the gut microbiota. An overabundance of pathogenic bacteria in the human gastrointestinal tract can elicit a pro-inflammatory response in the host and lead to the development of inflammation associated with dysbiosis, such as colitis (Wang et al., 2022). Deterioration of colonic inflammation can result in death from septic shock. Probiotics are defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host. The precise taxonomy of probiotic strains is essential to their well-recognized beneficial effects. The genus *Lactobacillus* has emerged as a valuable and diversified reservoir of probiotic species. Most *Lactobacillus* species are facultative anaerobes; a few are obligate anaerobes. They are catalase-negative, fermentative with the production of lactic acid as a major end product from carbohydrate metabolism. The important species within the *Lactobacillus* genus are *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, and *Lactobacillus rhamnosus*. Species of the *Lactobacillus* genus are metabolically active in the intestines of humans and other animals and are closely related to human health.

II.2.6.3. Anti-inflammatory Effects

In the gut, the balance between inflammatory and anti-inflammatory stimuli is critical for maintaining intestinal immune homeostasis. Under physiological conditions, mucosal tolerance prevents chronic and unnecessary inflammation against non-pathogenic antigens, such as food and commensal microbiota. However, intestinal inflammation may occur when the balance is disrupted by risk factors such as stress, environmental toxins, changes in microbiota composition, and diet. This inflammation is characterized by increased levels of pro-inflammatory cytokines, epithelial cell apoptosis, and aberrant modulation of immune responses due to dysbiosis and impaired mucosal integrity. Chronic intestinal inflammation is believed to facilitate the development of metabolic disorders

(Concetta Cufaro et al., 2023). Therefore, therapies aimed at restoring homeostasis are needed, including dietary interventions to modulate microbiota composition.

Probiotic bacteria, defined as viable microorganisms that exert beneficial health effects, are considered a promising dietary strategy against chronic inflammation. In particular, lactic acid bacteria (LAB) are widely used as human probiotics and foods. LAB are non-pathogenic, generally recognized as safe, and have been linked to immune modulation. *Lactobacillus plantarum* is a prominent LAB in fermented foods that has beneficial effects and is applied as a food supplement to prevent or alleviate metabolic disorder symptoms. *L. plantarum* strains are also considered a class of probiotics with potential health-promoting effects. It has been shown that most *L. plantarum* strains have enterococcal properties and could exert beneficial effects on gut-associated health parameters.

Several strains of *L. plantarum*, including WCFS1, Lp62, ZJ317, and C9O4, have shown anti-inflammatory effects aimed at gastrointestinal disorders after induction of inflammatory stimuli in HCT116 or Caco2 cells, in suspensions with THP-1-derived macrophages, or in mouse models. Investigated whether and how *Lpb. plantarum* C9O4 affects TNF-α and IFN-γ mediated inflammatory pathways in the inflammatory cell model Caco2 and the cytokine levels IL-6 and IL-8, damaged enterocyte biomarkers Caspase-3/GSK-3β/P38 MAPK, and intestinal barrier integrity in Caco2 and Caco2/THP-1 co-culture models.

II.2.7. Mechanisms of Action

Lactobacillus plantarum is endowed with a wide variety of probiotic functions, which differ according to strain. This diversity is probably reflected in the chemicals produced by this species, and this review intends to summarize and categorize the compounds produced by *L. plantarum* and their likely mechanisms of action, with special emphasis on the last decade's novel discoveries about the Lp-1 strain (Teresa Rocchetti et al., 2021). With probiotics, it is generally meant the microbial strains that confer a health benefit when administered in adequate amounts. Most of the probiotic bacterial strains studied are mostly belonging to the *Bifidobacterium* and *Lactobacillus* genera. However, recently new strains belonging to different genera are being explored as newly discovered probiotic strains, or due to new analysis on strains that were believed to be known for decades. The microbiota that colonizes the GIT contains a plethora of microbial species,

and only some of them have good colonization potential and are rewarded by the GI tract with substrates produced by the main specialists in polysaccharide degradation. Such species, aside from lactobacilli, are being explored for probiotic effects. The probiotic potential seems to reside not only in the chemicals produced by a given strain, but also in the biosynthetic gene clusters that are putatively involved in the biosynthesis of these chemicals. The genome of *L. plantarum* strains includes many secondary metabolites biosynthesis biosynthetic gene clusters, and there is the hope that strains to be explored for probiotic effects harbor such gene clusters but have not yet been examined. Besides the better-known antimicrobial compounds produced, which are generally small molecules, lactobacilli produce a plethora of other antimicrobial molecules, of different chemical nature, debt of attention under the probiotic point of view. Probiotics can exert their beneficial effects through a range of complementary mechanisms that may act separately or in concert. Adherence to intestinal tissues, production of antimicrobial factors against enteric pathogens, modulation of local immune responses and maintenance of gut health and integrity, improvement of the gut barrier, weaving vascular and nervous systems connected to the gut, effect on gut microbiota composition, interaction with pathogenic agents, are only some of the mechanisms that have been proposed for probiotic lactic acid bacteria.

II.2.7.1. Gut Microbiota Modulation

Gut microbiota of mammals consists of trillions of several microbial populations, which contribute positively to host physiology throughout life. Gut microbiota play key roles in host digestion, metabolism, nutrition, immune system development, pathogen exclusion, modulation of gut barrier function, and the gut-brain axis. Since the microbiota of healthy intestines and the altered microbiota of diseased intestines has a specific composition and different activity, modulation of gut microbiota through probiotics or prebiotics can prevent or alleviate intestinal and systemic disorders by restoring the dysbiotic gut microbiota. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. The most widely used probiotics are lactic acid bacteria and bifidobacteria, which are classified as GRAS candidates. Among lactic acid bacteria, *Lactiplantibacillus plantarum* strains are widely used as starter cultures in various fermented products and are used as probiotics (Teresa Rocchetti et al., 2021).

L. plantarum strains have attracted attention as probiotics due to their powerful antimicrobial activity against enteropathogens, inhibition of bacterial toxin generation, and beneficial effects on intestinal health. The antimicrobial activity of *L. plantarum* has been attributed to various organic acids, hydrogen peroxide, and bacteriocin. Bacteriocins produced by LAB include two main classes called class I and class II. Class I bacteriocins are known as lantibiotics, which are ribosomally synthesized and posttranslationally modified bacteriocins. Class II bacteriocins are membrane-active, small, and heat-stable peptides often containing nonmodified amino acids and disulfides. In a few cases, bacteriocin-producing *L. plantarum* strains have been investigated for in vivo and gastrointestinal tract protective effects against infection by pathogens. Such studies reported a possible role of bacteriocins as anti-infective agents in pathogenesis and detection. In this context, proteomic and transcriptomic approaches were used to dissect molecular mechanisms underlying beneficial properties. Preclinical studies with animals are time-consuming and labor-intensive experiments that have long been used to evaluate the efficacy of probiotic strains and their health-promoting properties in a systemic way.

II.2.7.2. Production of Antimicrobial Substances

Regular daily dose of probiotics has beneficial health effects, in part because probiotics produce antimicrobial substances. Examples of these antimicrobial substances are organic acids, and antimicrobial peptides or bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial peptides. Metabolites that lower pH, such as lactic acid and acetic acid, also have antagonistic effects against numerous microorganisms (Teresa Rocchetti et al., 2021).

Bacteriocins produced by lactic acid bacteria (LAB) are classified into four classes according to their structure, genetic origins, and physiochemical properties. Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) bacterial species and are widely present in fermented foods such as cheese, yogurt, and sausages as a starter culture. LAB offer potential applications in food safety and protection due to the production of bacteriocins with antimicrobial activity against pathogenic and spoilage bacteria. LAB bacteriocins, which exhibit antimicrobial activity against food-associated pathogens, include thermophilin, lactocin, *Lactobacillus gasseri* LG36 bacteriocin, *Lactobacillus helveticus*, pico/lactocin, and plantaris. Antimicrobial peptides with efficacious activity against pathogenic bacteria and viruses are utilized as a putative bio-preservative in many food products. Antimicrobial peptides, in particular bacteriocins, play an important role in

food safety and protection. They can inhibit pathogens in food products and are less toxic than synthetic preservatives.

Bioactive peptides derived from fermented cheese inhibit Listeria monocytogenes growth in curd. Lactobacillus paracasei is also used as adjunct cultures in low-fat Cheddar cheese to control the growth of Listeria monocytogenes. Traditional foods made with Lactic Acid Bacteria (LAB) are discussed as a source of novel anti-microbial peptides. This chapter discusses LAB fermented foods, bioactive peptides from LAB, health-promoting effects of LAB bioactive peptides, extraction methods and technologies of bioactive peptides, and bioactive peptide production from LAB fermented foods

II.2.8.1. Definition of Prebiotics

Prebiotics are defined as a non-digestible food ingredient that regulates the growth or activity of beneficial microflora in the gastrointestinal tract in order to improve the host health (K. Sivamani et al., 2023). Other terms have been used as synonymous with prebiotic: “bioactive oligosaccharides”, “bifidogenic oligosaccharides”, “non-digestible oligosaccharides”, “functional oligosaccharides”, or “fermentable oligosaccharides”. In order to be classified as a prebiotic, a food ingredient should be resistant to gastric acid, should hesitantly be fermented by the indigenous microflora, and should result in the production of short chain fatty acids (SCFA). Common prebiotics include inulin, oligofructose, and galacto-oligosaccharides (GOSs). More recently, other prebiotics have been identified including cocoa powder and coffee. Prebiotics accept fermentation by and selective stimulation of growth of the prebiotics, Lactobacillus, and Bifidobacterium. A variety of plant-derived oligosaccharides are known to stimulate the proliferation of human gut bacteria. Prebiotics are organic substances selectively analysed by the host’s bacterial flora. They contribute to the liveable ecosystem of the gut microbiota. They affect the bending relationship between the host and microorganisms. They have favourable metabolic effects on the host or its environment. The western diet lacks prebiotics include, generally non-digestible carbohydrate. But the consumption of a prebiotic high-fiber diet results in changes in the composition and/or functional activity of gut microbiota that lead to health benefits. This effects could be studied with defined prebiotic compounds added to a control diet as well with the analysis of component-rich prebiotics. The identification and characterization of new dietary prebiotics from pomegranate peel extracts as new functional foods could be studied. Effects of the pomegranate peel extracts on the gut microbiota, especially in the beneficial bacterium. The intention was to elaborately study

the prebiotic potential of pomegranate peel. It was less known that pomegranate peels showed prebiotic activity against human gut microbiota.

II.2.8.2. Mechanisms of Action

The pomegranate fruit and associated products have gained significant interest due to their health-promoting effects. Pomegranate peel, which accounts for the largest portion of pomegranate byproduct, is an underutilized resource. It is rich in polyphenols and flavonoids, making it an important natural antioxidant source. Pomegranate peel also has antimicrobial activity, inhibiting the growth of pathogenic bacteria, and is a good source of prebiotics, promoting beneficial gut bacteria such as Lactobacillus and Bifidobacterium (K. Sivamani et al., 2023). Pomegranate is growing in popularity and is rich in antioxidants, vitamins, polyphenols, and fibers. Dietary fiber plays a significant role in the food chain. Humans cannot digest dietary fibers, but fermentation by gut bacteria produces an array of bioactive compounds such as short-chain fatty acids. The antioxidants, vitamins, polyphenols, and fibers contained in pomegranates play a vital role in promoting human health, especially cardiovascular health, owing to the prostaglandin-like antioxidant substances present. Although the edible arils of pomegranates are consumed, the peel and the whiteish inner astringent skin are generally disposed of as waste.

The pomegranate fruit comprises about 50% peel and seeds, and only arils are consumed as food; thus, 50% byproduct is generated. Pomegranates have attracted increasing interest in diet-based prevention of chronic diseases, such as cancer, due to their high contents of antioxidants, vitamins, polyphenols, and dietary fiber. Pomegranate peel is rich in antioxidant compounds and functions as effective antibacterial agent. Due to the presence of polyphenols, these byproduct can be used as a colorant, stabilizer, and antioxidant in food as well as cosmetics.

The presence of dietary fibers such as insoluble fibers and soluble fibers or phenolic compounds results in the prebiotic effect. Prebiotics are the substances selectively fermented by gut microbiota. The pomegranate peel promotes the growth of beneficial gut bacteria Lactobacillus and Bifidobacterium, resulting in increased feed utilization and growth performance of broilers. Drinking-stable fermented pomegranate peel supplementation for short periods helps prevent gut dysbiosis; however, effects on gut homeostasis by gut bacteria from regular consumption have yet to be studied.

II.2.8.3. Effects on Gut Microbiota

Among the myriad benefits conferred by pomegranate, the most alluring may be its potential to combat inflammation and boost levels of beneficial gut bacteria. As many know, gut bacteria have a strong influence on the body, and an imbalance may be associated with diseases including acne, depression, and Alzheimer's disease. An imbalanced gut microbiome profile is characterized by a greater quantity of pro-inflammatory bacteria and a reduction in microbial diversity, which contributes to an impaired gut barrier and elevated levels of circulating lipopolysaccharides, potentially leading to systemic inflammation (K. Sivamani et al., 2023).

Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit", being non-digestible food components that travel to generalist gut bacteria such as Firmicutes. Their bacterial digestion generates short-chain fatty acids, which are absorbed into the systemic circulation and shape a healthy gut–body communication system through the gut–brain axis and other routes. Preservation of gut microbial diversity and Firmicutes phyla is paramount since certain class taxa are associated with beneficial physiology.

Our findings indicate that PD does indeed influence the overall gut microbiome. While there was a trend towards becoming less dissimilar over time, this effect was not statistically significant. There may be opportunities to explore further dosing regimens or biomarkers of greater sensitivity to capture more subtle shifts in microbiome. Trend magnitudes may also be further amplified with a larger cohort size or lower variability. While gut bacteria, prebiotics, and short-chain fatty acids form a resilient, adaptive, and evolutionarily-fit system, these also provide opportunities for therapeutic design of exogenous computed and engineered pro-pre-post-biotics. The knowledge gleaned from the fundamental study systems herein will endure as a basis for more ambitious approaches and costly technological advances.

II.2.9. Pomegranate Peel: An Overview

Pomegranate (Punica granatum) belongs to the family Punicaceae and is considered one of the best health beneficial fruits. It is native to the region from Iran to the Himalayas in northern India and widely cultivated throughout the Mediterranean region, tropical, and subtropical regions, and West Asia. This bright red fruit has 3 to 6 cm thick skin and a leathery pericarp. Pomegranate peel is the outer layer of this fruit and was found to contain

more nutritional health-promoting activities than the fruit itself (Azmat et al., 2024). The pomegranate peel contains a maximum amount of phytochemicals, such as polyphenols, amino acids, sugars, tannins, flavonoids, terpenoids, ellagitannins, and micronutrients (vitamin C) and numerous minerals, including calcium, magnesium, potassium, iron, selenium, phosphorus, and zinc, which play an important role in the maintenance of cellular antioxidant homeostasis to prevent a number of diseases. Pomegranate peel is normally considered waste material after juice extraction, which ultimately leads to environmental pollution. Due to the bioavailability of intact edible components, it can be considered a dietary bioactive and health-promoting compound with nutraceutical applications in the food and pharmaceutical industries. Pomegranate peel powder or extracts in whole or functional form may be incorporated into meat, dairy, baked products, sauces, soups, vegetable products, and salads to improve consumer health and quality of the food products.

The preparation methods, such as water and ethanol extraction, freeze-drying, and heat drying temperature treatment, extraction methods, drying duration, and temperature treatment are necessary to develop high-value pomegranate peel extract to search for any potential health benefits. This review is designed to comprehensively illustrate recent advancements in the scientific knowledge about the phenolic phytochemical profile and nutritional composition of pomegranate peel and peel extract. Further, it explores possible applications of pomegranate peel and peel extracted bioactive wastes as nutraceuticals and food preservatives in human and animal nutrition. Pomegranate peel extract decreased the pH of Yeo-Bio after 0.5% bioactive incorporation, while the same concentration sustained the pH above 4.5 until half-life at day 12 with good synergistic viability. 1%, 0.5%, and 0.5% of bioactive incorporation maintained the sensory acceptability and overall quality of Yeo-bio up to 21, 15, and 6 days of storage, respectively. It can be summarized that the prebiotic potential of pomegranate peel and its extract can maintain the viability of probiotic organisms and functional characteristics of bioyoghurt.

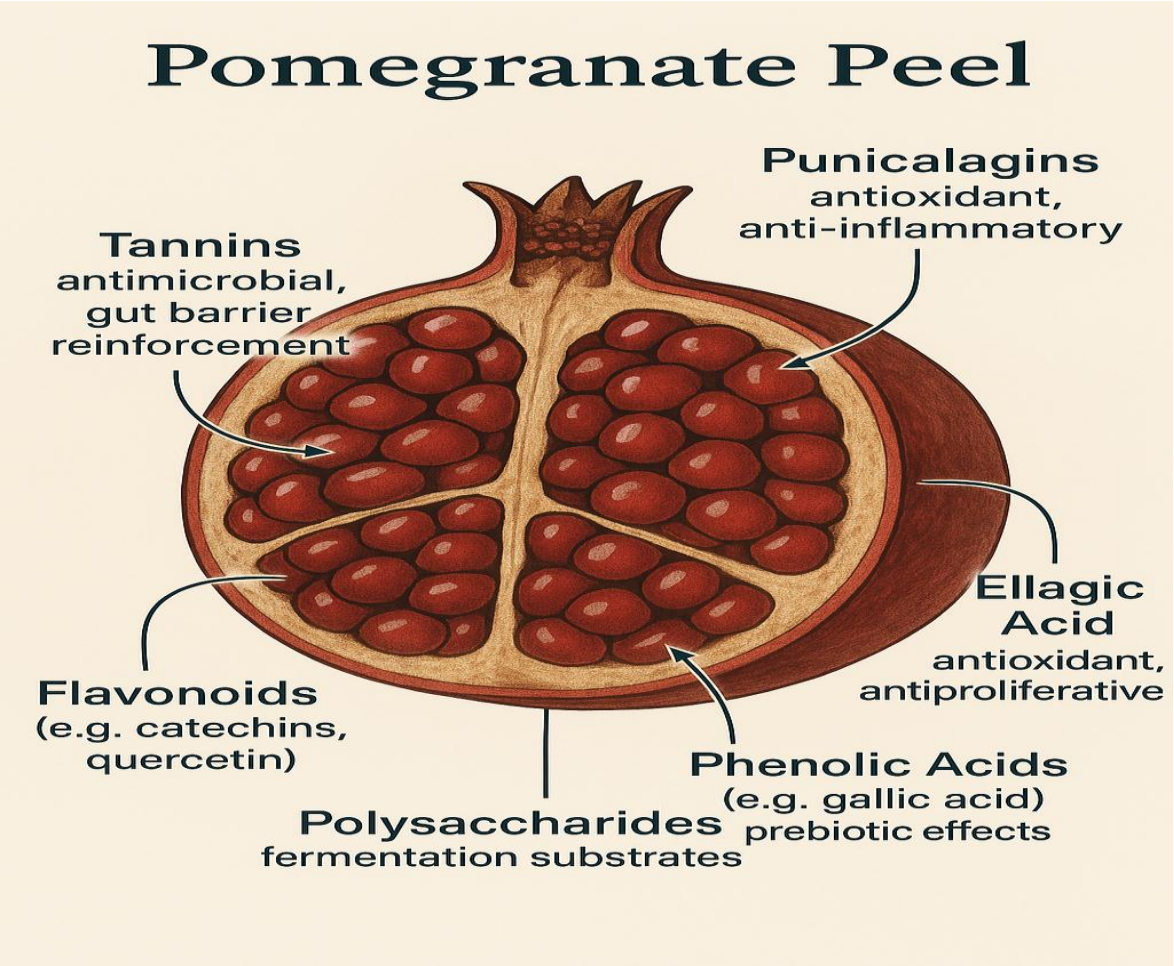


Figure 8: Bioactive Composition of Pomegranate Peel and Their Functional Roles in Gut Health

II.2.10. Nutritional Components of Pomegranate Peel

Pomegranates are recognized as a good source of dietary fiber, with the peels containing three times more than the arils. The dietary fiber content of dried peels is generally higher than that of fresh peels. Decoction and infusion of pomegranate peels can increase the dietary fiber content more than extraction by methanol, which suggests that soluble dietary fiber could be effectively extracted without or with a small amount of organic solvent (Azmat et al., 2024). The results of this study show that pomegranate peel extract (PPE) could be considered as a promising source of phenolic compounds with antioxidant activity. Pomegranate peel could be a good source of polysaccharides relevant to prebiotics.

II.2.10.1. Pectins

Pectins can be broadly categorized into high-methoxyl and low-methoxylpectins depending on their degree of esterification (HMPs and LMPs, respectively). The chemical properties of the gelling agents used need to be investigated before using pectins in the modification of food structure. In pectin's case, methyl esters (which are low-methoxyl) are typically used. Low-methoxylpectins are often considered to be less effective than high-methoxylpectins as they require a soluble calcium salt for gelation. In the presence of fruit juice constituents, it will contain divalent cations (to some extent) and thus have a good propensity to gel LMP-dextrins. Food structure also has an effect on transit time, with a slow transit time leading to increased satiety. While reducing food particle sizes, increasing mixing times, and hydrocolloid addition can all increase gel viscosity and slow gastric emptying, it is unclear whether this leads to altered ileal digesta viscosity as well. There is strong evidence that inhibition of gastric emptying will reduce appetite, but the effect of the viscosity of food entering the duodenum is much less well understood. Assessment of alterations to food structure is often conducted on food products in isolation, which is understandable due to the large number of variables involved in altering the structure of a food. However, digestion in vivo does take place in a complex mechanical and chemical environment in which the physical structure of food is altered by mastication and mixing with saliva and gastric fluids prior to entry into the small intestine. These changes can alter the number, structure, and concentration of food particles by which enzymes can access nutrients and by which nutrients and digestion products can access the intestinal epithelium (K. Sivamani et al., 2023).

II.2.10.2. Phenolic Compounds

Pomegranate peel (PP) is a kind of agricultural waste. The succulent red color arils are of great interest in the food industry as juice and garnishee. Though only 20-35% of the whole pomegranate fruit is eaten, the peel accounts for 45-70% of the fruit. To avoid waste, PP is often discarded or used as fuel, fertilizers, or animal feed. Since ancient times, pomegranate peels have always been used as folk medicines, owing to their numerous beneficial compounds. These bioactive compounds, such as tannins, flavonoids, and phenolic acids, contain a huge range of physiological activities, including antioxidant, anti-inflammatory, antidiabetic, antiobesity, antibacterial, antiviral, and intestinal microbiota modulation. It could alleviate the metabolic syndrome by decreasing blood glucose and cholesterol and increasing gut microbiota diversity and abundance of Butyricimonas. It is

more effective than regular feed additives like vitamin E, fiber, or diet-stimulated antioxidant substances (Mo et al., 2022).

Pomegranate peel is rich in healthy nutrients, for human health. Bioactive compounds, such as phenolic acids, flavonoids, and tannins, in the peels are more abundant than other parts of pomegranates. Complex bioactive compounds in pomegranate peel often exist in the form of a mixture, so the synergistic effect of different compounds can produce a variety of physiological activities. Recently isolated pure compounds with a well-studied structure are often well investigated in various aspects; however, bioactivity studies on complex compounds from natural products like raw extracts and ingredients are scarce. Pomegranate peel contains a variety of antioxidants, including vitamin C, hydrogen peroxide, tannins, flavonoids, and polyphenol compounds.

Phenolic compounds are the main secondary metabolites of the shikimic acid, pentose phosphate, and phenylpropanoid pathways and include a large number of water-soluble substances. A variety of pomegranate peel phenolic compounds are reported, among which tannins, flavonoids, and phenolic acids are the main ones. 94 phenolic compounds were identified in 12 pomegranate cultivars around the world. The amount of total phenolics in pomegranate peel differs by species, extraction solvents, and extraction methods. Individual phenolic compound content changes significantly even within the same species. The antioxidant effects are provided by polyphenol hydroxyl groups that can reduce the content of free radicals. Additionally, within a certain concentration range, PP could exert antioxidant activity and protect Caco-2 cells from oxidative stress by scavenging H2O2 and inhibiting intracellular ROS levels. Tannins may also exert antibacterial activity through the following mechanisms: inhibition of enzyme activity, precipitation of membrane proteins, and depletion of metal ions.

II.2.10.3. Vitamins and Minerals

Many studies have indicated that gut microbiota can be modulated and diversified by prebiotics. We investigated the modulation of intestinal microbiota by standardized pomegranate peel (PPe). Participants completed a 20-day diarrhea-monitoring diary and a 2-week washout period. During a 2-week double-blind study, participants were randomly assigned to consume either placebo or PPe. Fecal samples were collected and analyzed by high-throughput sequencing of the 16S rRNA gene to confirm the modulation of gut microbiota. At baseline, the gut microbiota was diverse and not correlated to gut

symptomibility. The test subjects exhibited a significant increase in Bacteroidetes and a decrease in Firmicutes after supplementation. F/B ratio, SCFA-producing bacteria (Roseburia and Ruminococcus), and anti-inflammatory bacteria (Bifidobacterium) exhibited a significant increase. Additionally, PPe supplementation enhanced several metabolite and microbial pathways and reduced harmful metabolite pathways. The prebiotic effect of PPe is highlighted by modulation of the intestinal microbiota and the production of SCFAs, providing useful information for understanding the dietary effects of PPe and improving gut wellness (K. Sivamani et al., 2023).

II.2.10.4. Antioxidants

Bioactive compounds of pomegranate, predominantly inside the peel, may act as natural antioxidants to protect against oxidative stress. Oxidative stress is the effect of excessive formation of reactive oxygen species (ROS) that cannot be eliminated by the antioxidant defenses of living organisms, resulting in pathological processes. Pomegranate peel is an abundant and economical source of bioactive compounds with antioxidant power. Additionally, bioactive constituents from the peel, such as polyphenols, tannins, and hormones, were found to be stable and functional after gastrointestinal digestion, exhibiting enhanced health-promoting abilities (Mo et al., 2022). Pomegranate peel extracts can scavenge free radicals and inhibit lipid oxidation. These extracts have been reported to prevent and treat nearly all diseases related to oxidative stress by enhancing the activities of superoxide dismutase, catalase, and glutathione peroxidase.

Currently, many studies focused on the biological activity of bioactive compounds derived from pomegranate peel; however, few studies mention their action mechanisms and bioavailability. Mechanisms of enhancing gut health by preventing and/or delaying the hyperplasia or crooked of mucus-secreting intestinal goblet cells, neutralizing the hypersecretion of mucus, and scavenging oxidative stress by polyphenols in pomegranate peel extracts were proposed. Bioactive compounds of pomegranate peel were also confirmed to protect against gut dysbiosis induced by antibiotics by restoring the population of probiotics and inhibiting the growth of pathogenic bacteria through non-covalent interaction, phenolic hydroxylysis, and hydrogen ionization with divergent targets (K. Sivamani et al., 2023). However, due to the different sources and extraction methods of bioactive compounds, variations in bioactive compound levels and antioxidant capacities were observed across different bioactive compound preparations. Therefore, bioactive compounds derived from pomegranate peels should be confirmed to give better extraction

conditions to obtain small molecular polyphenolic compounds with stronger antioxidant activities.

II.2.11. Prebiotic Properties of Pomegranate Peel

In the quest for health-promoting food sources, a special attention is paid to plants rich in polyphenols participating in a prebiotic effect (Azmat et al., 2024). One of such plants interesting for further studies is pomegranate (Punica granatum L.). Its peel and seed oil are widely investigated as valuable sources of biocompounds, food additives, and nutraceuticals. Pomegranate peel is rich in polyphenolic compounds, primarily pomegranate ellagitannins, whose prebiotic effect is under investigation for different sources. As with pomegranate peel, the pomegranates are widely described as prebiotic compounds. The detailed study of pomegranate peel as a prebiotic compound is limited, and it has the potential to be a unique prebiotic for yogurt. Pomegranate peel contains different polyphenolic compounds promoting beneficial microorganisms affecting the content of microbiota of bio-glucose yogurt package. In the past decade's pomegranate peel is significantly studied, and its nutritional composition is researched firstly using HPLC-PDA, where a higher amount of gallic acid, ellagic acid, and punicalagins is reported. The total phenolic and flavonoid contents of pomegranate peel are higher than the mentioned parts. The hemicelluloses' value is relatively higher in the pomegranate peel than in other fruits. The antioxidant activity of the pomegranate peel is strongly correlated with the high phenolic and flavonoid contents. Bioactive and jelly candies are prepared from it. Acetic acid bacteria (AAB) yeast are enriched. Compounded bio-yogurt is prepared from this widely available peel. Nutrilixir, a keto-caloric liquid formula, is prepared, which promotes beneficial microbes' growth in glucose-fortified milk systems. Moreover, research should focus on the beneficial microflora's growth in prebiotic-rich food substances that can mitigate health-related issues in the gut.

PART III. CHAPTER TWO

III. Chapter 2: Materials and Methods

III.1. Study Location

All the experimental work was carried out at the Laboratory of Microbiology, Faculty of Science and Technology, University of Saïda Dr. Moulay Tahar.

III.1.1. Culture Media

The culture media used were prepared in the form of broth, solid agar, or semi-solid agar. The media included:

- **GN medium**: a non-selective isolation medium used for the enumeration of total mesophilic flora.
- **MRS medium**: used for the isolation of lactic acid bacteria.
- **LB medium** (*Luria-Bertani*): used for the growth of Enterobacteriaceae.
- **Chapman medium**: a selective medium for halophilic bacteria, particularly *Staphylococcus* species.
- **VRBL medium** (*Violet Red Bile Lactose*): used for the detection and enumeration of coliform bacteria.
- **SS medium** (*Salmonella-Shigella*): a selective medium for the isolation of *Salmonella* and *Shigella* species from clinical or food samples.

III.2. In Vitro

III.2.1. Selection and Revivification of Probiotic Strains

In this study, two *Lactobacillus* strains with probiotic potential were selected. These strains were previously isolated and studied in earlier works by Amara S. from 2012 to 2020: *Lactobacillus plantarum* NSC10, isolated from camel milk in Naâma (Algeria), and *Lactobacillus plantarum* JUMIII4, isolated from mare's milk in Saïda (Algeria). Both strains were revived in 20 mL of liquid MRS medium and incubated at 37 °C for 72 hours. After incubation, each strain was streaked onto MRS agar and incubated again at 37 °C for 24 to 48 hours. Once well-isolated colonies were obtained, the macroscopic appearance of each strain was observed and confirmed through smear preparation for microscopic examination, allowing verification of the morphological characteristics specific to each strain (Amara et al., 2019).

III.2.2. Evaluation of the Probiotic Potential of the Selected Strains

III.2.2.1. Hemolytic Activity

Hemolytic activity was evaluated following the method described by Amara et al. (2019). The hemolytic character was assessed by streaking *Lactobacillus* strains onto Columbia blood agar. After 24 hours of incubation at 30 °C, the type of hemolysis was examined.

Hemolysis zones appear as follows: α-hemolysis (greenish halos), β-hemolysis (clear zones around colonies), or γ-hemolysis (no change in the medium) (Maragkoudakis et al., 2006).

III.2.2.2. Lipolytic Activity

To evaluate lipolytic activity, lactic acid bacteria were inoculated onto solid MRS agar adjusted to pH 7 using a 0.1 M phosphate buffer (Na_2HPO_4 /NaH_2PO_4). Tween 80 was incorporated at 1% (w/v) as the exclusive lipid source, and calcium carbonate ($CaCO_3$) was added at 0.5% (w/v) to opacify the medium, enhancing the visibility of enzymatic reactions. Bacterial cultures, grown for 18 hours, were spot-inoculated onto the prepared medium and left to dry at ambient temperature for one hour. Plates were then incubated at 30 °C for 48 hours. Lipolytic activity was identified by the formation of clear halos surrounding *Lactobacillus* colonies, with zone diameters measured post-incubation (Amara et al., 2019).

III.2.2.3. Proteolytic Activity

To assess proteolytic activity, MRS agar supplemented with 10% skimmed milk was poured, solidified, and dried. Lactic acid bacteria were then spot-inoculated from 18-hour-old cultures. After 24 hours of incubation at 37 °C, proteolysis was revealed by clear zones around the colonies. Larger halos indicated stronger proteolytic activity of the strains (Sadi et al., 2017).

III.2.2.4. Acid Tolerance

The method consisted of exposing the *Lactobacillus* strains to different pH levels ranging from pH 1 to pH 6. MRS broth series were adjusted to the respective pH values using 1 M HCl. Each tube was inoculated with 1% of a *Lactobacillus* culture ($OD_{600\,nm} \approx 1$). Bacterial growth was estimated

spectrophotometrically at 600 nm after 24 hours of incubation at 37 °C (Amara et al., 2019).

III.2.2.5. Bile Salt Tolerance

Tolerance to bile salts was evaluated at increasing concentrations (0.25%, 0.5%, 1.0%, 2.0%, 5.0%, and 10%). Sheep bile was sterilized using a Millipore membrane filter (Millipore, MILLEX-GV, 0.22 µm, SLGV0130S, Perkin Elmer, Boston, MA) and then aseptically added to MRS broth. Each tube was inoculated with 1% of a pre-culture of *Lactobacillus* ($OD_{600\,nm} \approx 1$). Bacterial growth was measured after 24 hours of incubation at 37 °C using a spectrophotometer at 600 nm (Amara et al., 2019).

III.2.2.6. Antibiotic Resistance

The antibiotic susceptibility of the strains was assessed using the disk diffusion method on agar medium, in accordance with the recommendations of the French Society for Microbiology's Antibiogram Committee (Bonnet et al., 2013). A bacterial suspension was prepared from an 18–24-hour-old culture in liquid MRS medium. Antibiotic discs (Erythromycin 60 µg, Rifampin 30 µg, Gentamicin 200 µg, Penicillin G 100 IU, Amoxicillin 30 µg, Vancomycin 5 µg) were aseptically placed on the surface of MRS agar previously inoculated with the bacterial culture using the swab technique. After 24 hours of incubation at 37 °C, inhibition zones around the discs were measured, and the strains were classified as Sensitive (S) or Resistant (R) according to the Committee's standards (Bonnet et al., 2013).

III.2.2.7. Antimicrobial Activity Against Pathogenic Bacteria

The objective of this test was to evaluate the inhibitory effect of lactic acid bacteria on pathogenic bacterial strains. Pre-cultures of both probiotic and indicator strains were prepared. *Lactobacillus* strains were grown in MRS broth, and pathogenic bacteria were cultured in LB broth enriched with 4% yeast extract to support the growth of all microorganisms.

Lactobacillus cultures were spot-inoculated onto solid enriched LB agar. After a drying period of 2 hours at room temperature, plates were incubated at 30 °C for 24 hours. The resulting colonies were then overlaid with 10 mL of semi-solid LB agar seeded at 1% (v/v) with a fresh culture of the

indicatorpathogenic strain. The antagonistic effect of *Lactobacillus* was evidenced by the formation of inhibition zones around their colonies, measured after 24 hours of incubation at 37 °C (Amara, 2019).

III.2.3. Plant Material

The plant material used in this study is pomegranate peel (*Punica granatum*). The pomegranates were purchased from the local market. Only ripe and intact fruits were selected for this study. To prepare the pomegranate peel powder, fruits from a cultivar with a yellowish color were first washed twice with distilled water. The peels were then manually removed. These samples were first dried at room temperature away from sunlight, then placed in an oven at 40 °C until their weight stabilized. The peels were then ground into powder using a mortar and sieved to obtain a particle size of 500 µm. The resulting powder was stored in a desiccator containing silica gel.

III.2.4. Extraction of Bioactive Compounds

The extraction was carried out using a reflux setup directly from 50 g of pomegranate peel powder added to 400 mL of distilled water and heated for two rounds of two hours each (Hachem et al., 2016).

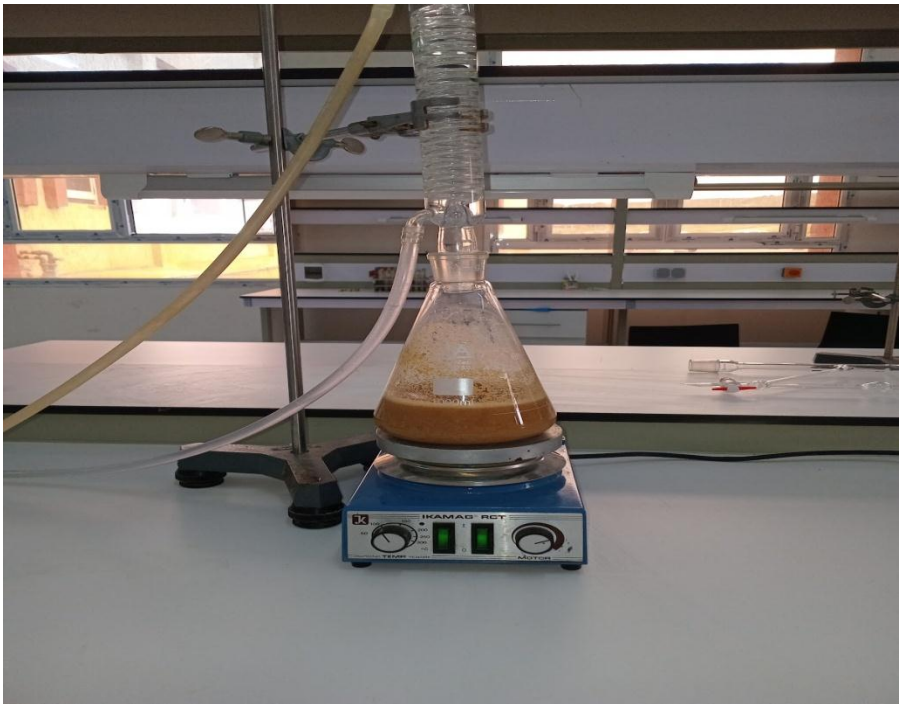


Figure 9: Reflux setup for aqueous extraction of pomegranate peel

III.2.5. Qualitative Analysis by Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was used to determine the sugar composition of wall polysaccharides (Randerath, 1971). These underwent two types of hydrolysis:

III.2.5.1. Partial Acid Hydrolysis

This method enables hydrolysis of matrix polysaccharides such as pectins using diluted acids, according to Harche et al., 1991.

III.2.5.2. TLC Procedure

One-dimensional chromatographic separation was performed on silica gel plates with reference sugars, solvent systems, and spray reagents as described.

III.2.6. Phytochemical Screening

A series of qualitative tests were conducted to identify the major families of bioactive phytochemicals present in the pomegranate peel extract.

III.2.6.1. Detection of Polyphenols

Using the ferric chloride reaction ($FeCl_3$): 2 mL of the extract mixed with one drop of 2% alcoholic $FeCl_3$. A bluish-black or dark green coloration indicates a positive result (Tuo, 2015).

III.2.6.2. Detection of Flavonoids

- *Ferrous Sulfate Test ($FeSO_4$):* Yellow coloration after adding diluted sulfuric acid suggests the presence of flavonoids (Arul & Sangeetha, 2015).
- *Sodium Hydroxide Test:* Yellow-orange color after adding NaOH 10% indicates flavonoids (Tuo, 2015).

III.2.6.3. Detection of Tannins

- *Ferric Chloride Test:* Blue-black or dark green precipitate with 1% $FeCl_3$ suggests hydrolysable tannins.
- *Ferrous Sulfate Test:* Green or dark brown color with 1% $FeSO_4$ indicates condensed tannins.
- *Gelatin Test:* A flocculent precipitate after adding 1% gelatin indicates tannin presence.

III.2.6.4. Detection of Alkaloids

Mayer's reagent added after acidification reveals a green or white precipitate if alkaloids are present (Arul & Sangeetha, 2015).

III.2.6.5. Detection of Coumarins

1 mL of extract mixed with 10% NaOH; yellow coloration indicates coumarins (Arul & Sangeetha, 2015).

III.2.6.6. Detection of Terpenoids

Brown-red interface layer after reaction with chloroform and concentrated sulfuric acid indicates terpenoids (Arul & Sangeetha, 2015).

III.2.6.7. Detection of Saponins (Foam Index Test)

Persistent foam after vigorous shaking and resting for 15 minutes reveals the presence of saponins (Tuo, 2015).

III.2.6.8. Detection of Steroids – Salkowski Test

A red coloration after adding concentrated $H_2\ SO_4$ suggests the presence of steroid compounds (Tuo, 2015).

III.2.6.9. Detection of Reducing Compounds

Formation of brick-red precipitate after boiling with Fehling's reagent indicates the presence of reducing sugars (EL-Haoud et al., 2018).

III.2.6.10. Detection of Carbonyl Compounds (Aldehydes/Ketones) – DNPH Test

The presence of aldehydes or ketones was confirmed by the formation of an orange-yellow precipitate upon reaction with dinitrophenylhydrazine in concentrated $H_2\ SO_4$ (Bhandary et al., 2020).

3. *In Vivo*

The intricate community of microorganisms residing in the gastrointestinal tract—collectively known as the gut microbiota—plays a pivotal role in host metabolism, immune modulation, and barrier integrity. Disruption of this balanced ecosystem (dysbiosis) has been implicated in a wide array of disorders,

from low- grade inflammation to metabolic syndrome and inflammatory bowel disease. Animal models, particularly Wistar rats, offer a controlled platform to explore how targeted microorganisms and prebiotics extracts can restore microbial equilibrium and attenuate inflammatory responses.

In this study, we designed a 21-day *in vivo* experiment to investigate the effects of:

- **Probiotics** (*Lactobacillus plantarum* strains NSC10 and JUMII4)
- **Prebiotic** (aqueous extract of pomegranate peel)
- **Synbiotic** combination (probiotics + prebiotic)

on gut microbial dynamics and systemic inflammation under induced intestinal stress. Aspirin administration on Day 18 serves as a mild pro- inflammatory stimulus, mimicking conditions of dysbiosis and barrier compromise.

Five batches of male Wistar rats (n = 5 per batch) will be compared:

1. **Batch 1 (Negative Control)**: standard chow & water only, no aspirin.
2. **Batch 2 (Vehicle Control)**: standard chow & water, water gavage + aspirin on D18.
3. **Batch 3 (Probiotic)**: standard chow & water, daily *L. plantarum* gavage + aspirin on D18.
4. **Batch 4 (Prebiotic)**: standard chow & water, daily pomegranate- peel extract gavage + aspirin on D18.
5. **Batch 5 (Synbiotic)**: standard chow & water, combined probiotic + prebiotic gavage + aspirin on D18.

Fecal samples collected at baseline (D1), mid-treatment (D7), pre-aspirin (D18), and pre-sacrifice (D21) will enable microbiota profiling, while terminal blood analyses (CBC, CRP) on Day 21 will quantify systemic inflammation. This protocol aims to elucidate how probiotic, prebiotic, and synbiotic strategies can modulate gut ecology and host immune status in the face of an inflammatory challenge.

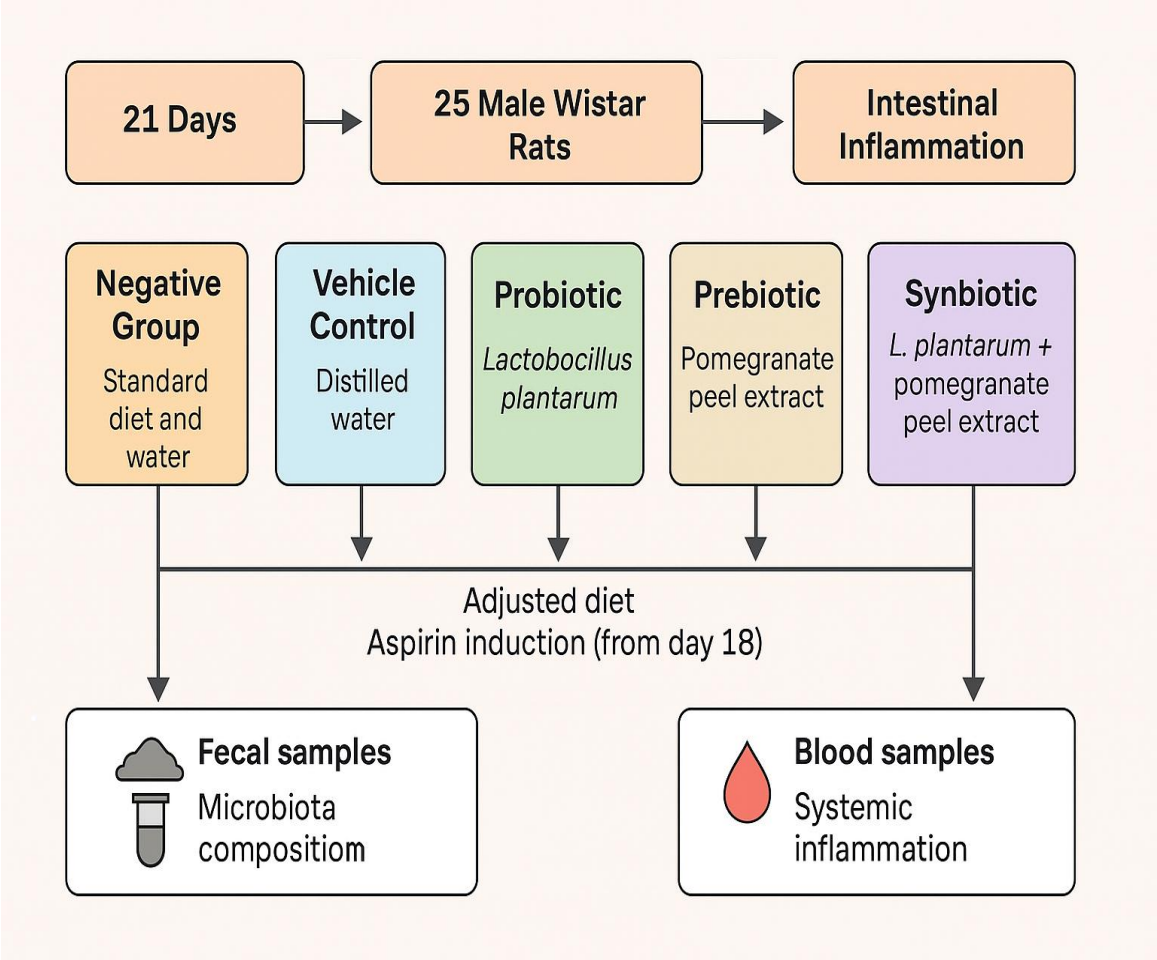


Figure 10: Diagram of the in vivo protocol evaluating the effects of probiotic, prebiotic, and synbiotic treatments on aspirin-induced intestinal inflammation in Wistar rats.

III.3.1. Housing and Feeding

The in vivo experiment was carried out at the Ain Hdjar animal facility (Department of Biological Sciences, Faculty of Natural and Life Sciences, Dr Moulay Tahar Saïda University). Twenty-five male Wistar rats, aged between 1 and 2.5 months, were housed in groups of five per polycarbonate cage on wood-chip bedding. Drinking water was provided ad libitum throughout the study. Each rat received a daily ration of 20 g of standard feed, adjusted individually to ensure consistent nutritional intake across all experimental groups.

Figure 11: Rat housing conditions

III.3.2. Preparation of the Strains

The two probiotic strains were each inoculated into 10 mL of MRS broth and incubated at 30 °C for 24 hours. The culture was then transferred into a 200 mL flask and incubated under the same conditions for an additional 24 hours. From this culture, 5 mL of each strain were harvested by centrifugation, the supernatant (MRS broth) discarded, and the bacterial pellet resuspended in 0,5 mL of sterile physiological saline (0.9 %). The suspension was vortexed briefly and administered to the rats by oral gavage.

Figure 12: Oral gavage administration to rats

III.3.3. Body Weight Monitoring and Dosing of Probiotics and Prebiotics

Each rat's body weight was recorded daily prior to treatment to monitor weight progression and ensure accurate dosing. The probiotic suspension, containing *Lactobacillus plantarum* strains NSC10 and JUMII4, was administered by oral gavage at a volume of 0.5 mL per rat per day from Day 1 to Day 17 for Batches 3 (Probiotic) and 5 (Synbiotic).

The aqueous pomegranate peel extract, used as a prebiotic, was administered to Batches 4 (Prebiotic) and 5 at a dose of 250 mg/kg/day, as recommended by Muhialdin et al. (2023). For rats weighing approximately 200 g, this corresponds to 50 mg of extract per rat per day. The extract was prepared by refluxing 50 g of peel powder in 800 mL of distilled water, yielding 30.42 g of dry extract. This resulted in a final concentration of 38.02 mg/mL. The required volume to deliver 50 mg per rat was calculated using the formula:

Volume (mL) = Dose (mg) / Concentration (mg/mL)Volume = 50 mg / 38.02 mg/mL ≈ 1.31 mL

Thus, each rat received 1.3 mL of extract per day from Day 1 to Day 17. For Batch 5, the synbiotic group, rats were administered 0.5 mL of probiotic suspension and 0.5 mL of prebiotic extract separately each day during the treatment phase.

III.3.4. Aspirin Suspension Preparation and Administration

To induce gastrointestinal inflammation, an aspirin suspension was prepared by opening 30 capsules to obtain 3 g of aspirin powder, which was transferred into a clean beaker. One milliliter of Tween 80 was added as a dispersing agent, followed by 40 mL of distilled water. The mixture was stirred thoroughly, then adjusted to a final volume of 100 mL by adding an additional 60 mL of distilled water. The suspension was homogenized to ensure uniform dispersion and stored at 4 °C. Prior to each administration, the solution was re-homogenized.

To standardize conditions, food was withdrawn 5 hours before aspirin administration and water was removed 2 hours prior to dosing. Food was reintroduced 1 to 2 hours post-administration. The dosage was adjusted according to the body weight of each rat, with individual volumes ranging from 0.51 mL to 1.02 mL, as detailed in **Table A1**

Figure 13: Aspirin Capsules Used for the Preparation of the Anti-inflammatory Suspension

Table 1: Individual Aspirin Dosing per Rat Based on Body Weight

Rat	Weight (g)	Dose (mg)	Volume Administered (mL)
Batch 2	155	22.23	0.775
	122	18.30	0.610
	102	15.30	0.510
	171	28.65	0.835
	204	30.50	1.020
Batch 3	193	22.65	0.785
	138	22.55	0.630
	131	22.65	0.755
Batch 4	157	22.65	0.755
	113	19.35	0.755
	188	26.20	0.940
	150	22.50	0.750

Rat	Aspirin Dose (mg)	Volume Administered
Rat 1	137	0.685
Rat 2	149	0.685
Rat 3	165	0.825
Rat 4	146	0.730
Rat 5	189	0.945

III.3.5. Microbiological Parameters

The assessment of intestinal flora was carried out using fecal samples collected at four key time points during the experiment: Day 1 (before treatment), Day 7 (after one week of gavage), Day 18 (prior to aspirin induction), and Day 21 (day of sacrifice). For each sampling, 5 grams of fecal matter were collected from different areas of the cage and homogenized in 45 mL of sterile physiological saline using a mortar and pestle. The resulting suspension was subjected to a series of decimal dilutions ranging from 10^{-1} to 10^{-6}. From each dilution, 1 mL was plated onto five specific culture media. Only the plates containing between 30 and 300 colony-forming units (CFUs) were considered for enumeration, based on the method of Zacconi et al. (1999), adapted by Amara (2020).

The targeted microbial genera, along with their respective culture media and incubation conditions, are listed in the table below:

Table 2: Culture Media and Incubation Conditions for Bacterial Enumeration

Culture media and incubation conditions used for bacterial enumeration

Bacterial Genus	Culture Medium	Incubation Temperature
Total mesophilic flora	GN	37 °C
Lactobacillus spp.	MRS	30 °C
Staphylococcus sp.	Chapman	37 °C
Enterobacteriaceae	VRBL	37 °C
Salmonella–Shigella	SS	37 °C

III.4. Sacrifice Procedure and Biological Sampling

Animal sacrifice was performed on Day 21, three days after the induction of intestinal inflammation by aspirin. Two rats were randomly selected from each group for biological sampling.

The animals were euthanized by rapid decapitation using a sterilized sharp instrument, in accordance with ethical guidelines for animal experimentation. Blood was collected immediately post-mortem by allowing it to flow freely into appropriate tubes.

The blood samples were distributed as follows: – EDTA tubes for hematological analysis (complete blood count – CBC) – heparin tubes for serum separation, used for the quantification of C-reactive protein (CRP), following centrifugation at 3000 rpm for 10 minutes.

These samples were used to evaluate the effects of treatments on the animals' inflammatory and hematological status.

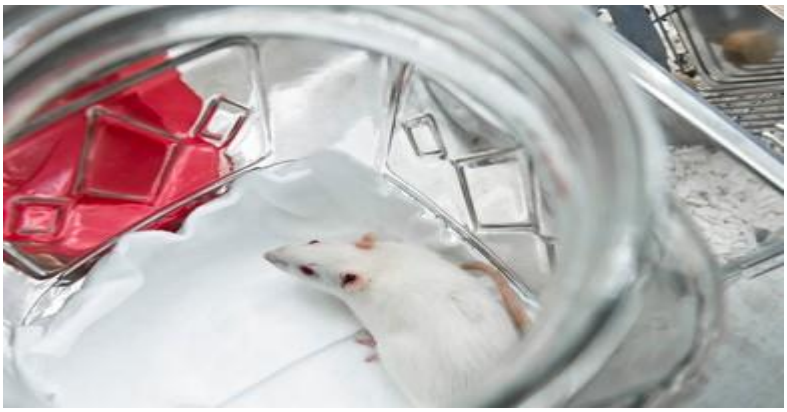


Figure 14: Subject Rat Prepared for Ethical Sacrifice and Blood Collection

PART IV. CHAPTER THREE

IV. Chapter 3: Results and Discussion

IV.1. In Vitro Characterization

IV.1.1. Lactobacillus plantarum NSC10 (Gx100)

Microscopic observations revealed that the lactic acid bacterial isolates, including *Lactobacillus plantarum*, predominantly appeared as Gram-positive bacilli arranged in chains or as single cells, which corresponds to the typical morphology of this species. These bacteria tested negative for catalase and oxidase activities, confirming their classification within the *Lactobacillus* genus.

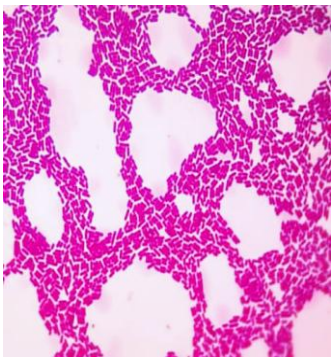


Figure 15: Microscopic observation of Lactobacillus plantarum NSC10 (Gx100)

IV.1.2. Lactobacillus plantarum JUMII4 (Gx100)

This strain exhibited the typical characteristics of *Lactobacillus plantarum*: Gram-positive bacilli, frequently observed in chains or singly, and negative for catalase and oxidase. These traits confirm its identification and taxonomic placement within *Lactobacillus* species.

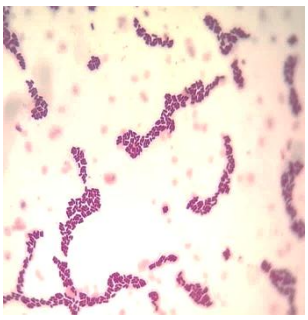


Figure 16: Microscopic observation of Lactobacillus plantarum JUMII4 (Gx100)

Figure 17: Streak inoculation from liquid MRS culture of Lactobacillus plantarum JUMII4

Lactobacillus plantarum JUMII4, when cultured on MRS agar, exhibited optimal growth at temperatures ranging from 30 °C to 37 °C, forming round, smooth colonies with a cream to white appearance—typical of *Lactobacillus* species. Growth was promoted by an acidic pH between 5.5 and 6.0, consistent with the selective composition of the MRS medium.

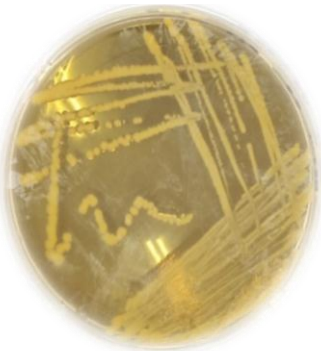


Figure 18: Streak inoculation from liquid MRS culture of Lactobacillus plantarum NSC10

IV.1.3. Hemolytic Activity :

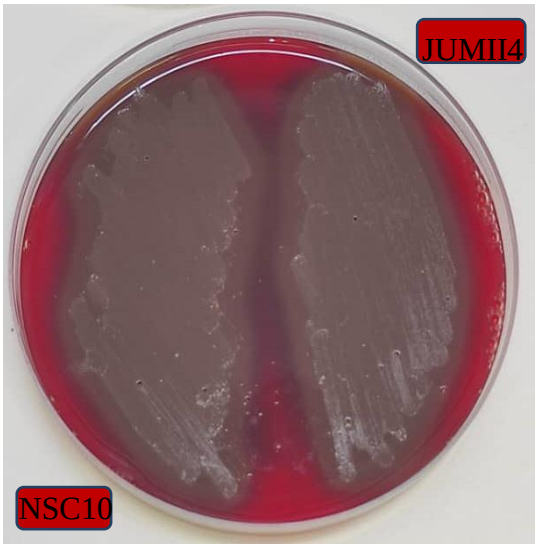


Figure 19: Hemolytic activity results of Lactobacillus plantarum NSC10 and Lactobacillus plantarum JUMII4

No hemolytic zones were observed for either *Lactobacillus plantarum* NSC10 or *Lactobacillus plantarum* JUMII4. This indicates that both strains do not degrade red blood cells, suggesting they are non-hemolytic and safe for probiotic use.

IV.1.4. Antibiotic Susceptibility of Lactic Acid Bacteria

This test aimed to assess the resistance of lactic acid bacterial strains to six antibiotics, providing insight into their safety profile and potential for therapeutic application.

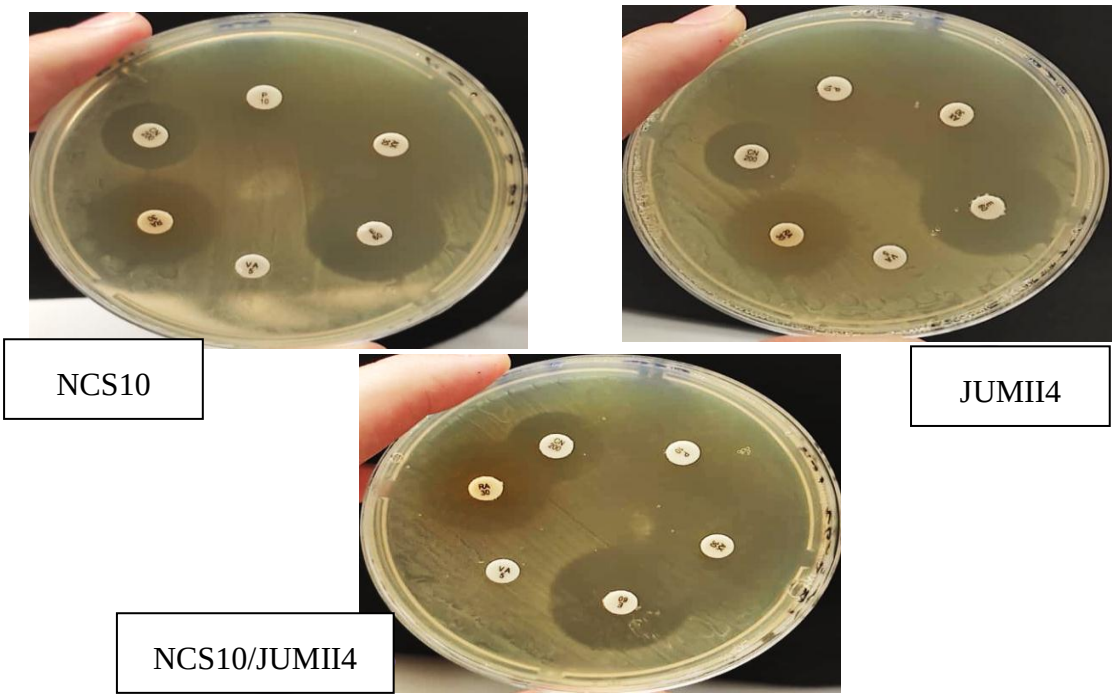


Figure 20: Antibiogram results of Lactobacillus plantarum NSC10 and Lactobacillus plantarum JUMII4

Table 3: Antibiotic Resistance Profile of JUMII4, NSC10, and Their Combination

ATB Souches	Amoxicillin S≥19≤R	Penicillin S≥26≤R	Vancomycin S≥12≤R	Erythromycin S≥22≤R	Rifampin S≥21≤R	Gentamicin S≥17≤R
JUMII4	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
NSC10	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
JUMII4+ NSC10	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible

R: inhibition zone diameter ≤ 26 mm **S**: inhibition zone diameter ≥ 26 mm

The antibiotic susceptibility profile of *Lactobacillus plantarum* strains NSC10 and JUMII4 revealed inhibition zones around the antibiotic-impregnated disks. However, the diameters of these zones were less than or equal to 2.5 cm, indicating reduced sensitivity to the tested antibiotics. A slight variation was observed between the two strains: JUMII4 exhibited marginally larger inhibition zones than NSC10, suggesting a slightly higher susceptibility. In the shared quadrant containing both NSC10 and JUMII4, the inhibition patterns remained consistent with those observed individually.

These results indicate that both *L. plantarum* NSC10 and JUMII4 possess either intrinsic or acquired resistance to amoxicillin, penicillin, and vancomycin. This phenomenon is commonly reported among certain *Lactobacillus* strains, which are often naturally resistant to antibiotic classes such as aminoglycosides and glycopeptides. Such resistance may be advantageous when these strains are used as probiotics, as it allows them to survive concurrent antibiotic treatments. However, caution is essential, as some resistance traits may be transferable—posing potential risks in clinical and food-related contexts. Therefore, it is crucial

to determine whether these resistances are chromosomally encoded (non-transferable) or plasmid-borne (potentially transferable)

IV.1.5. Bile Salt Resistance

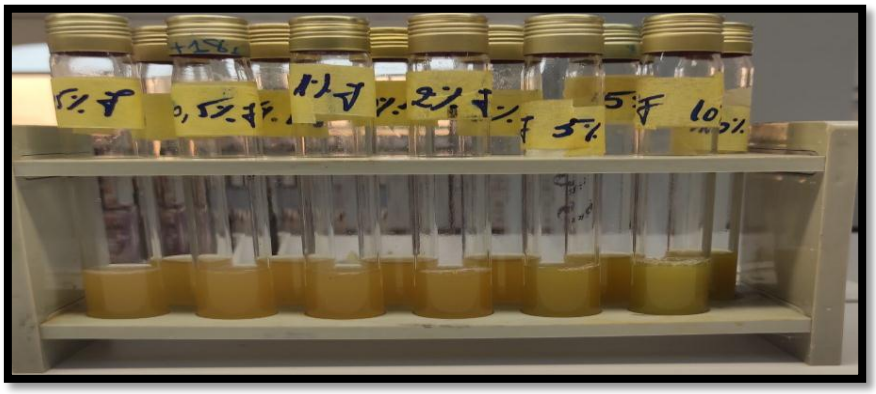


Figure 21: Bile salt resistance of Lactobacillus plantarum JUMII4 and Lactobacillus plantarum NSC10

Visible bacterial growth was observed in the test tubes, evidenced by turbidity in the medium at various bile salt concentrations (e.g., 5%, 10%). Growth varied depending on the concentration and, potentially, the strain used. Higher bile salt levels appeared to inhibit growth, indicating a threshold of bile tolerance.

The results show that the tested *Lactobacillus plantarum* strains (JUMII4 and NSC10) exhibited variable resistance to bile salts. This trait is crucial for survival in the digestive system, where bile salts exert strong selective pressure. Marked growth at 5% bile salts suggests good tolerance, while partial or complete inhibition at 10% indicates the resistance limit. These findings help identify the most promising probiotic strains, particularly those capable of withstanding intestinal stress conditions

Table 4: Growth of Lactobacillus plantarum Strains in the Presence of Different Bile Salt Concentrations

	0.25%	**0.50%**	**1%**	**2%**	**5%**	**10%**
NSC10	2.336	1.994	1.757	1.647	2.201	2.233
JUMIII4	2.410	1.919	1.723	1.587	2.005	2.295

Growth data presented in the table demonstrate good viability of both strains, even under high concentrations of bile salts, reflecting their tolerance to these stressful conditions.

At a low concentration (0.25%), both strains exhibited optimal growth, with optical density values of 2.336 for *Lactobacillus plantarum* NSC10 and 2.410 for *Lactobacillus plantarum* JUMII4. As bile salt concentration increased, a gradual decline in growth was observed up to 2%, indicating a dose-dependent inhibitory effect on bacterial proliferation. However, at 5% and 10%, growth increased again, which may be attributed to physiological adaptation or the activation of bile salt resistance mechanisms.

Strain JUMII4 showed overall greater tolerance to high bile salt concentrations, reaching a maximum optical density of 2.295 at 10%, compared to 2.233 for NSC10 at the same level. This may indicate a higher probiotic potential for JUMII4 under simulated intestinal conditions.

These findings suggest that both tested strains possess strong bile salt resistance—an essential trait for probiotics intended to survive and exert their benefits within the gastrointestinal tract.

IV.1.6. Acid Resistance

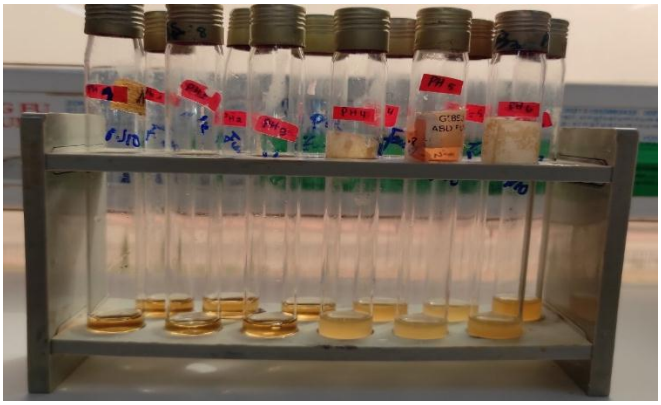


Figure 22: Acid resistance of Lactobacillus plantarum JUMII4 and Lactobacillus plantarum NSC10

Acid resistance is a key property for probiotic strains intended to survive transit through the gastrointestinal tract, particularly in the stomach, where the pH is extremely low. The results illustrated in Figure 8 show a comparison of acid resistance between two *Lactobacillus plantarum* strains: JUMII4 and NSC10.

Following incubation in an acidic medium, the culture tubes exhibited variable bacterial growth depending on the strain, observed as turbidity in the test tubes. *L. plantarum* JUMII4 demonstrated superior growth under acidic conditions compared to NSC10, indicating greater acid tolerance.

These results suggest that JUMII4 possesses more effective resistance mechanisms, such as intracellular pH regulation, production of acid stress proteins, or a more robust cell membrane capable of withstanding low pH environments. This trait gives JUMII4 a clear advantage as a probiotic strain, as it may better survive passage through the gastric barrier.

Table 5: Growth of Lactobacillus plantarum strains in MRS medium at different pH levels expressed as optical densities

	pH1	**pH2**	**pH3**	**pH4**	**pH5**	**pH6**
NSC10	0.050	0.074	0.163	2.435	2.726	2.833
JUMII4	0.060	0.081	0.196	2.474	2.678	2.690

The table reveals that *Lactobacillus plantarum* JUMII4 exhibits slightly higher growth than NSC10 across all tested pH levels, with modest differences in acidic environments (pH 1 to pH 4). For example, at pH 3, the optical density of JUMII4 reaches 0.196 compared to 0.163 for NSC10. At pH 2, the values are 0.081 and 0.074, respectively—showing only a minimal difference. These observations suggest that JUMII4 may have a slightly greater capacity to tolerate acidic conditions, potentially due to more efficient resistance mechanisms.

IV.1.7. Lipolytic Activity ResultsAfter 24 and 48 hours of incubation

Figure 23: Lipolytic activity results of Lactobacillus plantarum JUMII4 and Lactobacillus plantarum NSC10

Both strains, *Lactobacillus plantarum* JUMII4 and NSC10, exhibit lipolytic activity, as evidenced by the formation of clear zones around the wells. However, the clearance zone associated with the JUMII4 strain is slightly larger than that observed for NSC10, indicating more intense lipolytic activity in JUMII4. This may reflect the strain's enhanced ability to hydrolyze lipids, suggesting a stronger potential for fat degradation.

IV.1.8. Proteolytic Activity Results

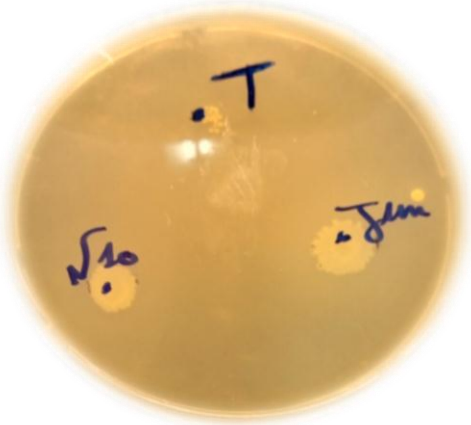


Figure 24: Proteolytic activity results of Lactobacillus plantarum JUMII4 and Lactobacillus plantarum NSC10

The results of the proteolytic activity for *Lactobacillus plantarum* strains JUMII4 and NSC10 show the formation of clear zones around the wells, indicating protein degradation. The JUMII4 strain exhibits a slightly larger clearance zone than NSC10, suggesting a more pronounced proteolytic activity. This reflects a higher enzymatic capacity of JUMII4 to break down proteins.

IV.1.9. Antagonistic Activity Results

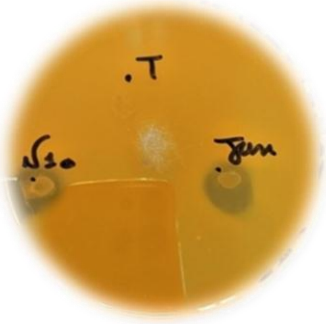


Figure 25: Antagonistic activity of JUMII4 against Bacillus subtilis

The Petri dish illustrates the inhibitory effect of the JUMII4 strain against *Bacillus subtilis*. A clear zone of inhibition surrounds the JUMII4 application site, indicating strong antimicrobial activity. NSC10 shows a smaller inhibition zone, suggesting comparatively weaker antagonistic potential.

Table 6: Diameter of inhibition zones (mm) for NSC10 and JUMII4 against selected pathogens

Pathogen	NSC10 (mm)	JUMII4 (mm)	Dominant Strain
Bacillus subtilis	10	16	JUMII4
Klebsiella pneumoniae	8	14	JUMII4
Bacillus cereus	9	15	JUMII4
Escherichia coli	7	13	JUMII4
Staphylococcus aureus	6	12	JUMII4

The antagonistic activity tests revealed that **JUMII4 consistently outperformed NSC10** across all tested pathogens. The inhibition zones were significantly larger around JUMII4, indicating a **stronger antimicrobial effect**, likely due to the production of **organic acids, bacteriocins, or hydrogen peroxide**.

This enhanced activity suggests that JUMII4 may be more effective in **suppressing pathogenic bacteria**, making it a promising candidate for **probiotic applications**. Its ability to inhibit *E. coli*, *K. pneumoniae*, *S. aureus*, and *B. cereus* highlights its potential role in **gut microbiota modulation and infection prevention**.

IV.2. Plant Material

IV.2.1. Phytochemical Screening of Phenolic Compounds

Tableau 7: Phytochemical Screening Results for Phenolic Compounds

	Présence / Absence
Plyphénol	++
Flavonoide	+++
Tannis C	+
Tannis H	++
Tannis G	++
Terpenoide	++
Alcaloide	-
Composés reducteur	+
Stéroide	++
Coumarines	+

Key: – = Absent, + = Slightly present, ++ = Moderately present, +++ = Strongly present

The results highlight a notable richness in **flavonoids and polyphenols**, suggesting potent antioxidant properties. The absence of alkaloids also reinforces the potential safety of the extract for further application.

IV.2.2. Thin-Layer Chromatographic (TLC) Profile of Pectic Oligosaccharides

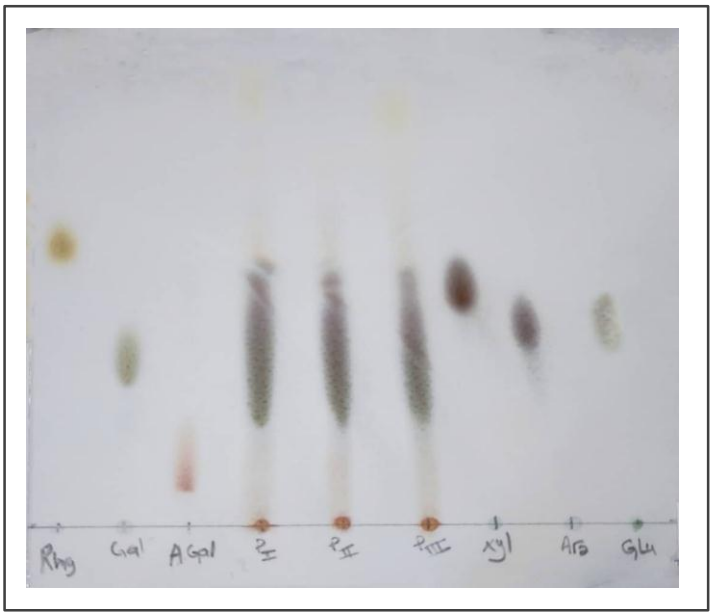


Figure 26: [N1] Thin-Layer Chromatographic (TLC) Separation Profile of Pectic Oligosaccharides

The qualitative analysis revealed the presence of xylose, arabinose, rhamnose, and galactose, suggesting the occurrence of rhamnogalacturonan.

General Observation Phytochemical screening of the pomegranate bark extract revealed a richness in bioactive compounds, notably flavonoids (+++), polyphenols (++), steroids (++), tannins (condensed, hydrolyzable, and gallic), terpenoids, and coumarins. Thin-layer chromatography (TLC) analysis highlighted the presence of simple sugars such as xylose, arabinose, rhamnose, and galactose, suggesting the presence of rhamnogalacturonan, a complex polysaccharide derived from pectins.

Discussion – Correlation with the Prebiotic Potential of Pomegranate Bark The combined findings from the phytochemical screening and thin-layer chromatography (TLC) analysis demonstrate that pomegranate bark is a rich source of bioactive compounds and pectic oligosaccharides with promising **prebiotic potential**.

The presence of abundant **flavonoids (+++)**, **polyphenols (++)**, and various forms of **tannins** (condensed, hydrolyzable, and gallic), along with **coumarins**

and **terpenoids**, suggests strong **antioxidant and antimicrobial properties**. These compounds are known to influence gut microbial balance by inhibiting pathogenic bacteria while supporting the growth of beneficial strains such as *Lactobacillus* and *Bifidobacterium*—a foundation of effective prebiotic action.

The TLC results confirmed the presence of **xylose, arabinose, galactose, and rhamnose**, key sugar residues of **rhamnogalacturonan**, a pectic polysaccharide. These sugars are components of **non-digestible oligosaccharides** capable of reaching the colon intact, where they can be selectively fermented by beneficial microbiota. This fermentation leads to the production of **short-chain fatty acids (SCFAs)** like acetate, propionate, and butyrate—essential metabolites that promote gut health, epithelial integrity, and local immune modulation.

Altogether, these results suggest that pomegranate bark extract offers a **dual biofunctional effect**:

- A **direct protective action**, via its antioxidant and antimicrobial phytochemicals;
- An **indirect prebiotic role**, through its fermentable oligosaccharides that support a healthy gut microbiome.

Such attributes highlight the potential of this natural extract for inclusion in functional food formulations or synbiotic systems that pair prebiotics with probiotic strains like *Lactobacillus plantarum*. Further in vivo investigations will help confirm its efficacy and safety as a prebiotic enhancer.

IV.3. In VivoStudy

IV.3.1. Body Weight

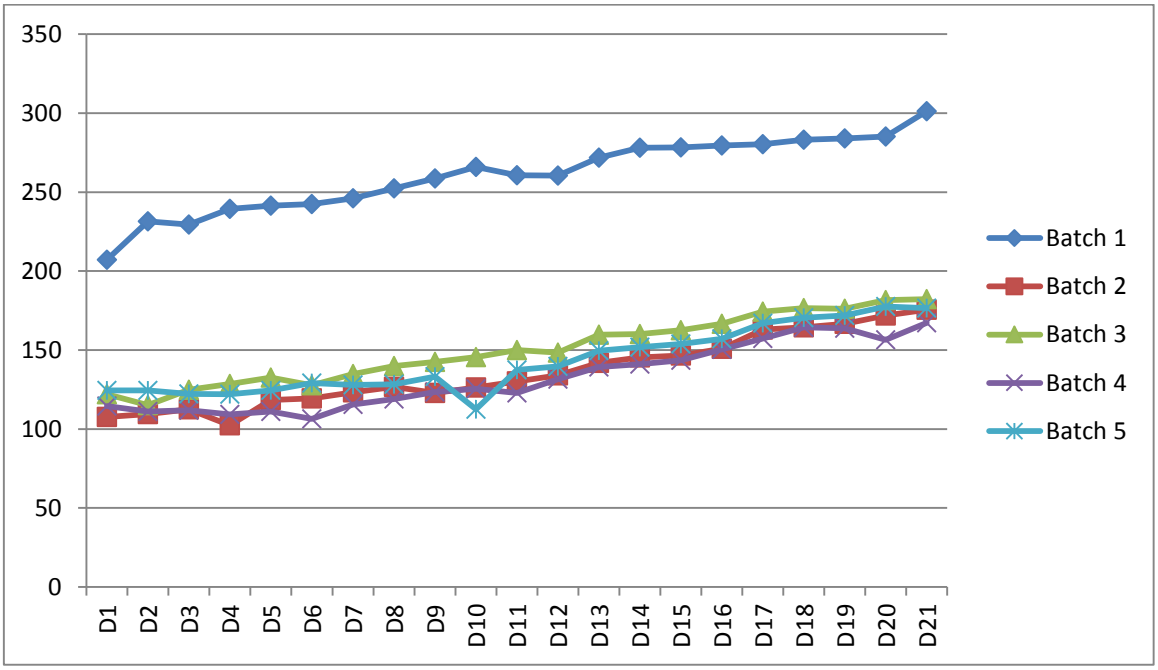


Figure 27: temporal evolution of rat body weight during the study

Over the 21-day experimental period, all groups displayed gradual weight gain, though with distinct trajectories:

- **Batch 1** (*negative control — older and heavier animals at baseline*) exhibited consistently higher body weight throughout the study, starting around 200 g and reaching approximately 300 g by Day 21. This expected growth occurred in the absence of any treatment or supplementation.
- **Batch 2** (*vehicle control — treated with distilled water*) showed the lowest weight gain among the treated groups, reflecting the absence of nutritional or microbiotic stimulation.
- **Batch 3** (*probiotic-treated group*) demonstrated a slightly higher weight gain than Batch 2, suggesting a modest physiological benefit potentially linked to probiotic supplementation.
- **Batch 4** (*prebiotic-treated group*) displayed a similar trajectory to Batch 2, with moderate weight gain, indicating a limited restorative effect.
- **Batch 5** (*symbiotic treatment: probiotic + prebiotic*) achieved one of the strongest gains among the treated groups, with weight progression approaching that of Batch 1. This suggests a potential **synergistic effect** of the combined treatment, enhancing metabolic support and overall physiological resilience.

Interpretation

- The evolution of body weight across the five experimental batches reflects the physiological influence of administered treatments rather than the impact of inflammation, which was induced only during the final three days of the study. As expected, **Batch 1** (*healthy negative control*), composed of older and heavier animals, exhibited the highest weight gain throughout the study, serving as a reference for normal growth in the absence of supplementation.
- In contrast, **Batch 2** (*vehicle control receiving distilled water*) displayed the lowest weight trajectory among the treated groups, likely due to the absence of any microbiotic or nutritional stimulation. **Batch 3** (*probiotic-treated*) showed a slightly higher weight gain than Batch 2, suggesting a modest physiological benefit of *Lactobacillus plantarum*, potentially through microbial modulation or improved nutrient assimilation. **Batch 4** (*prebiotic-treated*) followed a similar trend, with moderate weight gain reflecting a limited prebiotic effect.
- Notably, **Batch 5** (*symbiotic treatment*) achieved the most substantial improvement among the treated groups, with weight curves approaching those of the healthy control. This suggests a potential **synergistic effect** between probiotic and prebiotic components, enhancing metabolic support and overall physiological resilience.
- These findings support the therapeutic interest of symbiotic combinations in promoting growth and metabolic balance. They also align with the hypothesis that gut-targeted interventions can influence systemic outcomes—an insight with valuable implications for microbiota-focused research.

IV.3.2. Body Temperature Monitoring

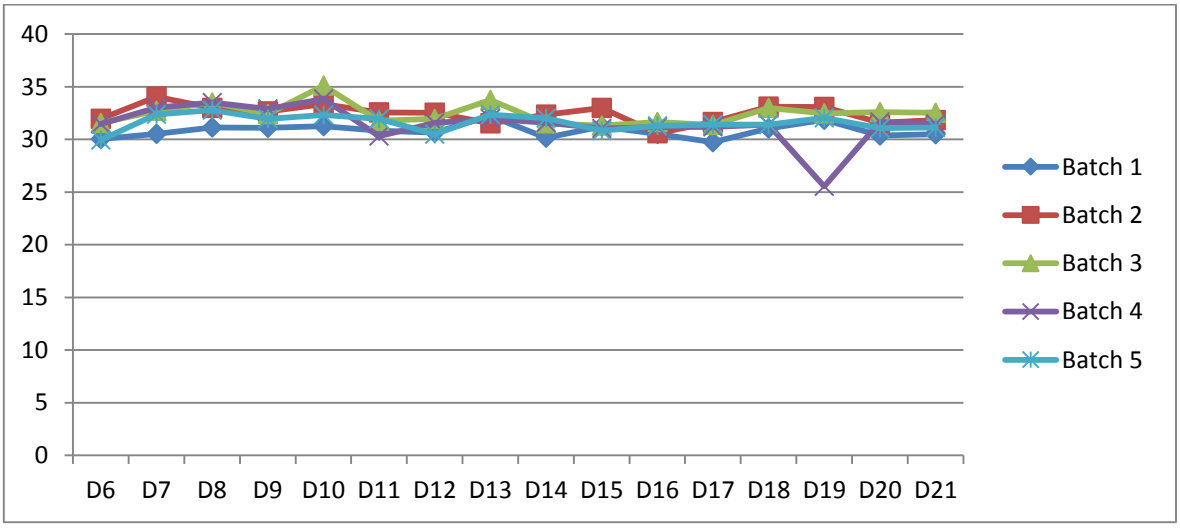


Figure 28: Temporal progression of temperature in rats throughout the study period.

General Observation on Body Temperature (Batch 1 to Batch 5) Throughout the observation period (D6 to D21), the body temperatures of all five groups remained relatively stable, oscillating within a narrow range between 30°C and 35°C, suggesting a return to physiological homeostasis. However, one

notable deviation was observed in Batch 4 on day 19 (D19), where a transient drop in temperature occurred—distinct from the other groups. Aside from this anomaly, the curves of the remaining batches exhibited only minor fluctuations.

Interpretation The stability of body temperature across most batches indicates that the animals maintained thermoregulatory balance, despite the induced inflammatory challenge. Batch 1 (negative control) consistently displayed higher and more stable temperatures, as expected in non-inflamed healthy animals. In contrast, Batch 2 (positive control receiving distilled water) sustained a slightly lower but steady temperature, consistent with systemic stress or inflammation and the absence of protective treatment.

Batch 3 (probiotic-treated) and Batch 5 (symbiotic-treated) showed fairly stable profiles comparable to the control, which may reflect a protective or anti-inflammatory role of the administered probiotic and symbiotic formulations. Their ability to maintain body temperature suggests reduced physiological impact of inflammation or improved systemic regulation.

The transient dip in Batch 4 (prebiotic-treated) on D19 may suggest a short-lived systemic response or external stress, possibly metabolic in origin. However, since the temperature normalized shortly after, the effect appears to be reversible or non-pathological.

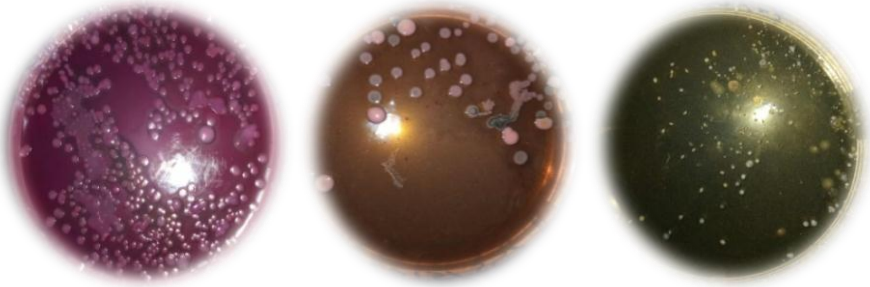
Overall, the data support the hypothesis that symbiotic and probiotic treatments may enhance host resilience by modulating inflammatory effects and contributing to systemic stability—even under induced stress.

IV.3.3. Microbiological Parameters

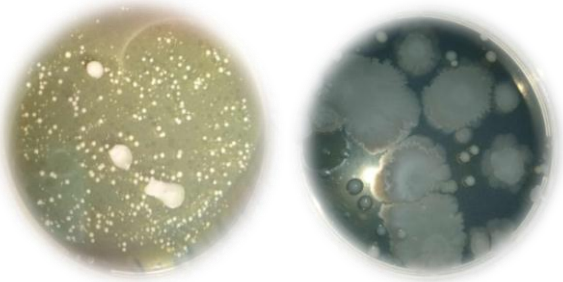
The enumerated fecal microbiota included total mesophilic flora, Enterobacteriaceae, lactic acid bacteria, and staphylococci. Selective and differential media were employed to isolate and quantify these microbial groups:

- **GN (GéloseNutritionnelle – Nutrient Agar):** Used for the enumeration of total mesophilic aerobic bacteria, which may include genera such as *Bacillus*, *Pseudomonas*, and *Micrococcus*.
- **VRBL (Violet Red Bile Lactose Agar):** Selective for *Enterobacteriaceae*, including genera like *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, and *Proteus*.

- **MRS (de Man, Rogosa and Sharpe Agar):** Favorable for the growth of lactic acid bacteria (LAB), particularly *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus*.
- **Chapman (Mannitol Salt Agar):** Selective for staphylococci, primarily *Staphylococcus aureus* and *Staphylococcus epidermidis*, due to their high salt tolerance.



1: VRBL 2: SS 3: MRS



4: Chapman 5: GN

Figure 29: Culture Media Used for the Enumeration of Fecal Microbiota

IV.3.3.1. Total Mesophilic Flora

The enumeration of total mesophilic flora on GN medium revealed notable variations in bacterial load across experimental groups and time points. At baseline (J1), all batches exhibited comparable microbial levels, indicating a uniform initial state prior to treatment or inflammatory induction.

As the experiment progressed, Batch 1 (*negative control*) maintained the highest and most stable CFU counts, reflecting a balanced and resilient gut microbiota in the absence of external stress or supplementation. In contrast, Batch 2 (*vehicle control receiving distilled water*) showed a gradual decline in total bacterial load, likely due to the absence of microbiotic support and the acidic impact of aspirin administered during the final phase.

Batches 3, 4, and 5, which received probiotic, prebiotic, and symbiotic treatments respectively, exhibited lower overall mesophilic counts compared to Batch 1. This reduction is not indicative of microbial loss, but rather of microbial modulation: the administered *lactic acid bacteria* (LAB) produce lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins, which suppress non-lactic bacterial populations. The effect is particularly evident in Batch 4, where prebiotic supplementation alone resulted in the lowest counts by J21, suggesting limited conservation of microbial diversity under inflammatory conditions.

Batch 3 showed moderate conservation of microbial viability, while Batch 5 demonstrated a delayed but more balanced microbial profile, with CFU counts approaching those of the healthy control by J17. This trend supports the hypothesis that symbiotic supplementation enhances the colonization and activity of LAB, which in turn modulate the broader microbial ecosystem. The inverse relationship between LAB abundance and non-lactic bacterial load underscores the selective pressure exerted by probiotic metabolites.

These findings highlight the conservatory role of gut-targeted interventions in maintaining microbial equilibrium, rather than restoring it post-inflammation. They also reinforce the potential of symbiotic strategies to promote microbial resilience through biochemical modulation**.**

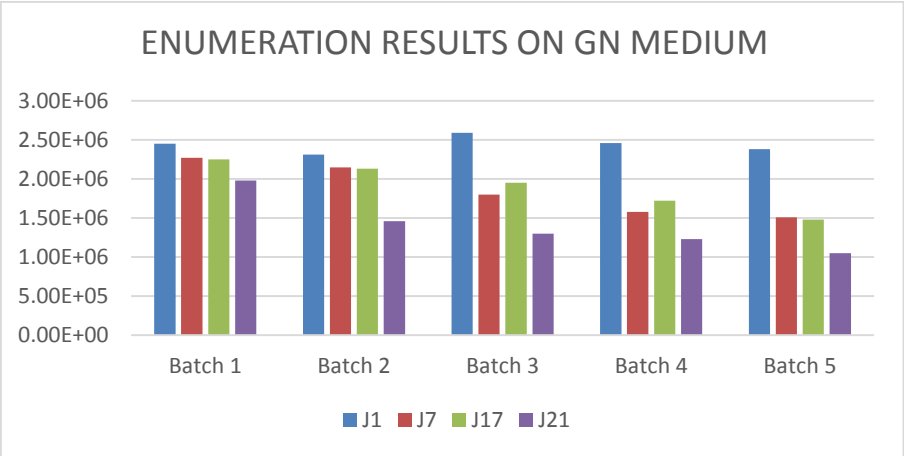


Figure 30: Evolution of Enterobacteriaceae Load on GN Medium Across Batches

IV.3.3.2. Enterobacteriaceae

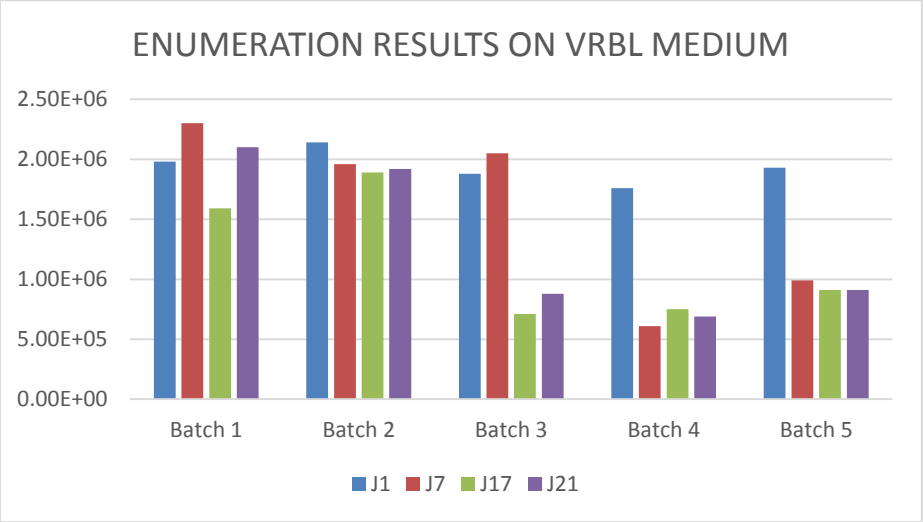


Figure 31: Evolution of Enterobacteriaceae Load

The quantification of Enterobacteriaceae on VRBL medium revealed distinct trends across experimental groups, reflecting the impact of both inflammation and microbiota-targeted interventions. Initially (J1), all batches exhibited comparable bacterial loads, confirming uniform pre-treatment conditions. However, a sharp rise was observed in Batch 2 (positive control treated with distilled water) by day 7, indicating a dysbiotic shift likely induced by inflammation and unopposed bacterial proliferation. This overgrowth of facultative anaerobes is consistent with intestinal barrier disruption and altered host-microbe interactions under inflammatory stress.

In contrast, Batch 1 (negative control) maintained stable and relatively lower Enterobacteriaceae levels throughout the study, reflecting healthy microbial homeostasis. Batch 3 (probiotic-treated) and Batch 4 (prebiotic-treated) showed moderate increases post-induction but demonstrated gradual normalization over time, suggesting a partial modulatory effect on microbial composition. Remarkably, Batch 5 (symbiotic-treated) maintained the lowest Enterobacteriaceae counts after J7, approaching those of the negative control group. This may reflect a synergistic suppressive effect on opportunistic Gram-

negative bacteria, likely mediated through competitive exclusion, pH modulation, or enhanced colonization resistance.

Altogether, these findings support the hypothesis that probiotic and especially symbiotic treatments can attenuate inflammation-induced microbial imbalances, reinforcing gut barrier function and limiting the expansion of potentially pathogenic taxa.

IV.3.3.3. Evolution of Lactic Acid Bacteria

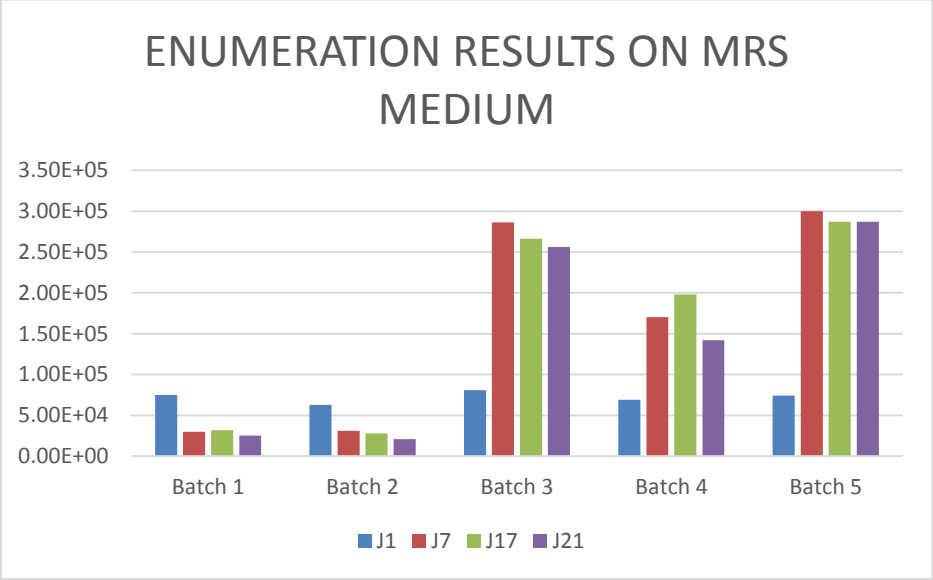


Figure 32: Evolution of Lactic Acid Bacteria

The enumeration of lactic acid bacteria (LAB) on MRS medium provided valuable insights into the microbial dynamics shaped by gut-targeted treatments and inflammatory conditions. At baseline (J1), all experimental groups presented comparable LAB loads, reflecting a homogenous initial state prior to intervention.

Over time, **Batch 1** (*negative control*) maintained moderate but stable LAB counts, confirming microbial equilibrium in the absence of inflammation or supplementation. In contrast, **Batch 2** (*positive control*) showed persistently low LAB levels throughout the study, illustrating the suppressive effect of inflammation when left untreated.

Batch 3 (*probiotic-treated group*) exhibited a marked and sustained increase in LAB counts, particularly from J7 onwards, reflecting the colonization and proliferative capacity of the administered strain, likely *Lactobacillus*

plantarum. **Batch 4** (*prebiotic-treated*) showed modest increases, suggesting that the prebiotic substrate selectively supported indigenous LAB growth to a limited extent. Notably, **Batch 5** (*symbiotic-treated*) recorded the highest LAB levels by J21, demonstrating a cumulative and synergistic effect of combined probiotic and prebiotic administration.

Importantly, the elevated LAB counts in treated groups (Batches 3, 4, and 5) are accompanied by a **replacement of pathogenic flora**. LAB produce **lactic acid**, **hydrogen peroxide (H_2O_2)**, and **bacteriocins**, which inhibit non-beneficial microbial populations. This microbial shift explains why **total mesophilic flora remained elevated** even under dysbiotic conditions: the increase reflects beneficial LAB dominance rather than pathogenic overgrowth.

These findings support the hypothesis that probiotic and symbiotic strategies not only enhance LAB proliferation but also contribute to **microbial rebalancing**, potentially improving gut barrier integrity and resistance to dysbiosis through competitive exclusion and biochemical modulation.

IV.3.3.4. Evolution of StaphylococcalLoad

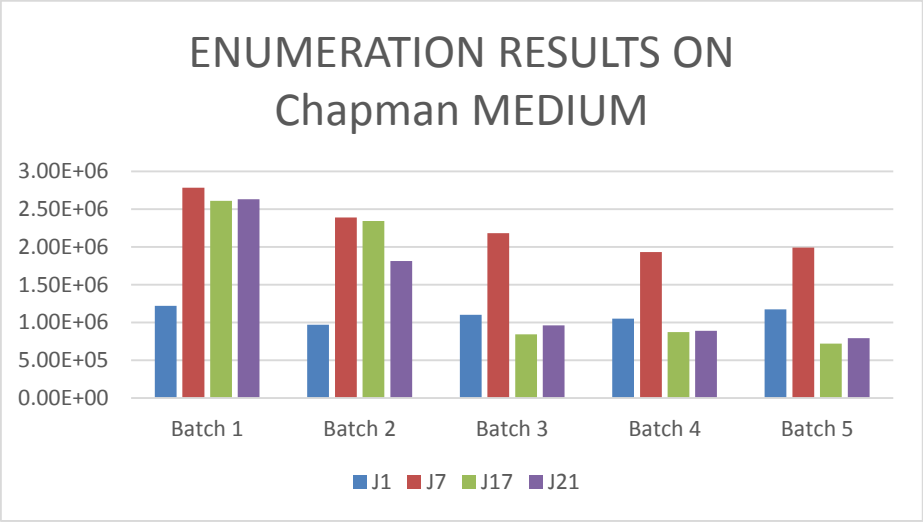


Figure 33: Evolution of Staphylococcal Load

The enumeration of staphylococci on Chapman medium revealed notable distinctions among experimental groups, reflecting the dynamic impact of inflammation and treatment on potentially pathogenic Gram-positive bacteria. At day 1 (J1), all batches exhibited similar bacterial loads, indicating an equivalent

microbial baseline. Over time, **Batch 2 (positive control)** recorded the **highest staphylococcal counts**, particularly at J17 and J21, suggesting that the inflammatory state favored the overgrowth of salt-tolerant opportunistic species such as *Staphylococcus aureus*. In contrast, **Batch 1 (negative control)** maintained stable and moderate levels throughout the study, underscoring microbial equilibrium in the absence of experimental stress.

Batch 3 (probiotic-treated) showed a moderate rise at J7, followed by a decline, possibly indicating a **transient imbalance followed by regulatory recovery** mediated by probiotic action. **Batch 4 (prebiotic-treated)** exhibited fluctuating levels, with no clear suppressive effect observed by J21, suggesting limited influence on staphylococcal proliferation. Strikingly, **Batch 5 (symbiotic-treated)** maintained consistently lower counts at later time points, highlighting a potential **synergistic inhibitory effect** against opportunistic staphylococcal species, likely through competitive exclusion, pH modulation, or enhanced immune-mediated control.

These findings suggest that while inflammation promotes the proliferation of opportunistic bacteria such as staphylococci, **symbiotic treatments may counteract this effect more effectively than probiotics or prebiotics alone**, reinforcing their value in managing inflammation-associated microbial disturbances.

IV.3.3.5. Evolution of *Shigella* and *Salmonella* Load

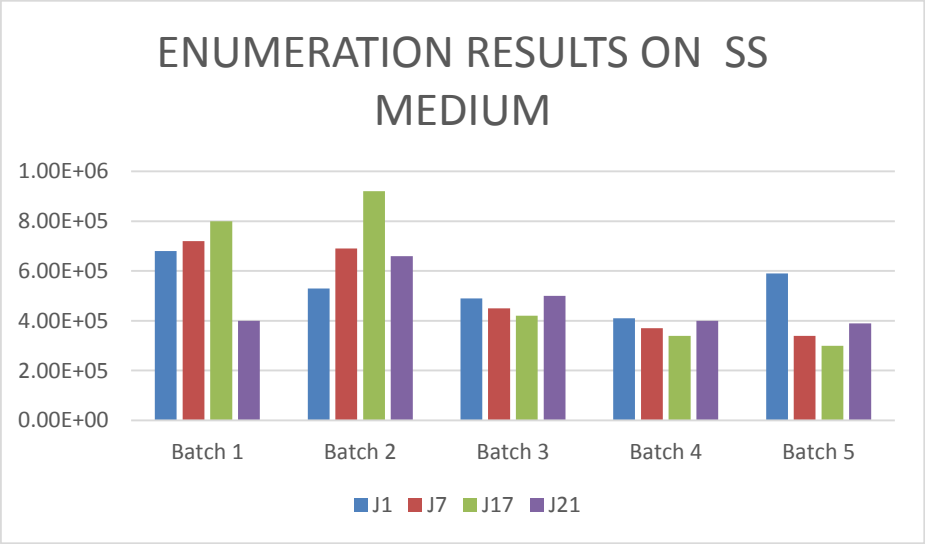


Figure 34: Evolution of Shigella and Salmonella Load

Enumeration on SS medium revealed important variations in the proliferation of enteropathogenic *Shigella* and *Salmonella* species under inflammatory stress and treatment conditions. At baseline (J1), all groups showed negligible or undetectable levels, indicating a balanced gut environment before induction. By day 7 (J7), **Batch 2 (positive control receiving distilled water)** presented a sharp increase in pathogenic load, with peak levels persisting at J17—suggesting inflammation-induced vulnerability and uncontrolled expansion of Gram-negative enteric pathogens.

In contrast, **Batch 1 (negative control)** maintained very low or undetectable counts throughout the trial, reflecting a stable, non-dysbiotic microbiota. **Batch 3 (probiotic-treated)** and **Batch 4 (prebiotic-treated)** exhibited transient elevations at J7 and J17, followed by a marked decline by J21, suggesting a **partial suppressive effect** against enteropathogen overgrowth. Notably, **Batch 5 (symbiotic-treated)** maintained minimal pathogen levels across all time points, especially by J21, indicating a **strong protective or competitive exclusion effect** likely mediated through microbial modulation and barrier reinforcement.

These results highlight the therapeutic relevance of microbiota-targeted interventions in suppressing opportunistic pathogens. The synergistic efficacy of symbiotic treatment in limiting *Salmonella* and *Shigella* colonization further reinforces its potential in inflammation-associated dysbiosis control.

Discussion

The analysis of fecal microbial dynamics across the various sampling time points highlights the evolving interaction between microbiota-targeted interventions and aspirin-induced inflammation. On day 1 (J1), before any treatment administration, microbial loads were comparable across all groups, confirming a balanced intestinal ecosystem. After 7 days of treatment (J7), initial shifts became noticeable: treated groups showed early signs of lactic acid bacterial stimulation, while pathogenic populations remained low, indicating the onset of beneficial microbial modulation. At day 17 (J17), following 17 consecutive days of probiotic, prebiotic, or symbiotic administration but prior to

induction, treated groups—particularly the symbiotic group—displayed reinforced levels of beneficial microbes and suppression of opportunistic bacteria, suggesting a well-established **preventive microbial shield**. Finally, at the sacrifice point (after 3 days of aspirin induction), the microbiota of pre-treated animals demonstrated **marked resilience**, with preserved total flora, elevated LAB counts, and reduced loads of Enterobacteriaceae, *Staphylococcus*, *Shigella*, and *Salmonella*, particularly in the symbiotic group. In contrast, the untreated positive control exhibited a pronounced dysbiosis, marked by overgrowth of facultative pathogens and depletion of beneficial taxa. These findings reinforce the value of sustained preventive strategies, especially the **symbiotic approach**, in preserving gut microbial balance under inflammatory stress.

IV.4. Hematological and Biochemical Analyses

As part of this study, biological analyses were carried out to assess the inflammatory status of the animals. Two types of markers were evaluated: **C-reactive protein (CRP)**, serving as a sensitive **biochemical indicator of systemic inflammation**, and the **complete blood count (CBC)**, with particular focus on **the total white blood cell (WBC) count** and the **percentage of granulocytes (GRAN)**—used to approximate neutrophil activity. Together, these parameters provide a reliable overview of the **presence or absence of an inflammatory process**, enabling a comparative assessment of how each treatment influenced the immune response triggered by aspirin.

IV.4.1. C-ReactiveProtein (CRP):

Table 8: Evolution of C-Reactive Protein (CRP) levels across experimental batches

Table X. CRP Levels in Each Experimental Batch

Batch	CRP Level (mg/L)
Batch 1	1.56
Batch 2	1.58
Batch 3	1.71
Batch 4	1.57
Batch 5	1.65

aspirin induction) revealed nuanced inflammatory profiles across the experimental groups.

Batch 2 (*negative control: no induction or treatment*) presented a CRP value of 1.56 mg/L, confirming the absence of inflammation and serving as the baseline reference for physiological conditions.

Batch 1 (*positive control: aspirin without treatment*) also recorded 1.56 mg/L, indicating a mild inflammatory response that did not exceed physiological thresholds—likely due to the short duration of aspirin exposure.

Batch 3 (*probiotic-treated*) exhibited the highest CRP level (1.71 mg/L), suggesting that the combination of aspirin and probiotic-derived lactic acid may have exacerbated the inflammatory response. Similarly, Batch 5 (*symbiotic-treated*) showed an elevated CRP value (1.65 mg/L), reflecting a comparable effect, possibly intensified by the synergistic activity of probiotics and prebiotics.

In contrast, Batch 4 (*prebiotic-treated*) recorded a CRP level of 1.57 mg/L, close to the baseline, indicating less pronounced inflammation. This may be

attributed to the non-acidic nature of prebiotics, which do not contribute to the acidic environment that can aggravate inflammatory pathways.

While CRP levels remained within a narrow range across all groups, these subtle differences suggest that probiotic-containing treatments (Batches 3 and 5) may have inadvertently intensified inflammation under aspirin-induced conditions, whereas prebiotic supplementation alone exerted a gentler modulatory effect.

IV.4.2. White Blood Cell (WBC) Count Analysis:

Table 9: Evolution of total white blood cell (WBC) counts across experimental batches.

Table X. White Blood Cell (WBC) Counts by Experimental Batch

Batch	WBC ($\times 10^9$/L)
Batch 1 (Control)	6.75
Batch 2	8.90
Batch 3	11.15
Batch 4	10.30
Batch 5	8.35

The total white blood cell (WBC) counts assessed at the end of the protocol (following 3 days of aspirin-induced inflammation) revealed distinct immunological profiles across experimental groups.

Batch 1 (*positive control: inflammation without treatment*) exhibited a WBC count of 6.75 $\times 10^9$ /L, serving as a reference for unmodulated inflammatory response. In contrast, Batch 2 (*negative control*) recorded a higher count of 8.90 $\times 10^9$ /L, which may reflect physiological variability rather than an inflammatory challenge, as no induction was performed.

Batch 3 (*probiotic-treated*) and Batch 4 (*prebiotic-treated*) displayed elevated WBC levels (11.15 $\times 10^9$ /L and 10.30 $\times 10^9$ /L, respectively), suggesting immune stimulation or reactive leukocytosis potentially linked to treatment effects. The increase in WBCs may also be influenced by the acidic environment created by probiotic metabolites such as lactic acid, which could accentuate inflammatory signaling.

Notably, Batch 5 (*symbiotic-treated*) demonstrated a lower WBC count (8.35 $\times 10^9$ /L) compared to Batches 3 and 4, indicating a more regulated immune

response. This supports the hypothesis that symbiotic supplementation offers superior modulation of inflammation, likely due to the balanced interaction between probiotic and prebiotic components.

Overall, while WBC levels remained within a moderate range, the data suggest that symbiotics are more effective in reducing inflammation than probiotics or prebiotics administered separately. These findings warrant further correlation with GRAN percentage and CRP levels to build a more integrated understanding of systemic immune modulation.

IV.4.3. Granulocyte (GRAN%) Profile Analysis:

Tableau 10: Evolution of Granulocyte Percentage Across Experimental Batches

Table X. Granulocyte (GRAN) Levels by Experimental Batch

Batch	GRAN (%)
Batch 1 (Control)	5.20
Batch 2	7.10
Batch 3	6.50
Batch 4	8.50
Batch 5	7.65

Observation – Granulocyte Percentage (%) Granulocyte levels, assessed at the endpoint of the protocol (after 3 days of aspirin-induced inflammation), offered insight into the **neutrophil-driven inflammatory response** in each group. **Batch 1 (positive control, no treatment)** displayed the **lowest GRAN percentage (5.20%)**, suggesting a surprisingly muted neutrophilic response despite aspirin administration. **Batch 2 (negative control)** showed a **slightly elevated level (7.10%)**, likely within physiological variance. **Batch 3 (probiotic-treated)** recorded **6.50%**, while **Batch 4 (prebiotic-treated)** showed the **highest**

percentage (8.50%), indicating possible **immune activation** in response to prebiotic fermentation products.

Importantly, **Batch 5 (symbiotic-treated)** registered a **moderate level (7.65%)**, higher than the positive control but lower than the prebiotic group, suggesting a **more balanced immune modulation**. Overall, these results reflect that the **granulocyte response varied subtly among treatments**, with symbiotic supplementation potentially offering **tempered neutrophilic recruitment** while maintaining immune readiness.

Discussion – Inflammatory Biomarkers Response

The combined evaluation of CRP, WBC, and granulocyte percentage reveals key insights into the systemic inflammatory status across experimental groups. **CRP**, a sensitive acute-phase protein, remained relatively stable among groups, but subtle variations revealed a trend. The **highest CRP value was observed in the probiotic group**, while the **symbiotic and prebiotic groups showed moderate reductions**, suggesting partial anti-inflammatory modulation, albeit not sufficient to fully counteract the inflammatory insult. Intriguingly, **the positive control group exhibited a CRP level identical to the negative control (1.56 mg/L)**, possibly reflecting a low-grade, aspirin-induced inflammation that did not exceed the baseline threshold in this parameter alone.

In contrast, **WBC counts** displayed more pronounced fluctuations. Both the **probiotic and prebiotic groups exhibited elevated leukocyte levels**, indicative of heightened immune activity or reactive leukocytosis. However, the **symbiotic group demonstrated a more tempered WBC response**, approaching that of the negative control, which may signal a better-regulated immune adaptation. When examined in tandem, **granulocyte percentages (GRAN%)** complemented this trend: the **prebiotic group recorded the highest neutrophil percentage**, potentially reflecting microbial fermentation by-products triggering innate responses. Meanwhile, the **symbiotic group maintained an intermediate GRAN%**, reinforcing the hypothesis of **balanced immune engagement** rather than suppression or overactivation.

Taken together, these findings suggest that while none of the treatments completely neutralized the inflammatory effects of aspirin, **symbiotic supplementation offered a more coherent modulation across all markers**—limiting excessive leukocyte recruitment, tempering neutrophil activation, and subtly reducing CRP levels. These immunological trends align well with your microbial observations, where the symbiotic group also demonstrated the most favorable modulation of intestinal flora. A cross-analysis of both microbial and systemic profiles strongly supports the hypothesis that symbiotics offer synergistic benefits in managing inflammation and gut ecosystem stability

CONCLUSION

CONCLUSION

This segment of the study demonstrated that aspirin-induced intestinal inflammation in Wistar rats led to significant microbial and systemic disruptions. Beneficial bacteria declined, while opportunistic and pathogenic strains—including Enterobacteriaceae, Staphylococcus, Shigella, and Salmonella—proliferated. These microbial shifts were accompanied by elevated inflammatory markers such as CRP, WBC, and GRAN, confirming systemic immune activation.

Microbiota-based treatments, particularly the symbiotic combination of camel milk-derived probiotic strains and pomegranate peel extract, showed a marked ability to restore microbial balance. This approach promoted the recovery of lactic acid bacteria, suppressed harmful flora, and helped regulate immune responses. Among all groups, the symbiotic treatment exhibited the strongest protective effect—maintaining ecological stability, physiological resilience, and immune control despite the induced inflammation.

These findings reinforce the potential of targeted microbiota modulation as a preventive strategy against inflammation-induced dysbiosis. The combined use of natural bioactives and resilient probiotics may offer a promising avenue for gut health support, warranting further investigation in clinical and molecular contexts.

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APPENDICES

VI.1. Appendix 1: Recorded Weights of Experimental Batches

DAY	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
D1	207,2	107,6	122	114,5	124,4
D2	231,4	109,4	115,2	111	124,4
D3	229,4	112,6	124,8	112	122,2
D4	239,4	102,4	128,6	109,3	122
D5	241,4	118,4	132,6	111	124,4
D6	242,4	119,4	127,8	106,5	129,2
D7	246	123,2	134,8	115,5	128
D8	252,4	126,8	139,8	119	128,4
D9	258,6	122,8	142,6	123,5	133,2
D10	266	126,2	145,6	125,5	112,6
D11	260,6	130,2	150	122,8	137,4
D12	260,4	134	148,4	131,5	139,6
D13	271,8	141,8	159,8	139,3	149,6
D14	278,2	145,4	160,2	141	151,8
D15	278,3	146,6	162,6	143,5	153,8
D16	279,6	150,8	166,6	150,5	157,2
D17	280,4	163	174,4	157,3	167
D18	283,2	164,4	176,6	164,3	170,4
D19	284	166,6	176,2	163,8	171,8
D20	285,2	172	181,6	156,5	177,6
D21	301,25	175,6	182,2	167,3	176,6

VI.2. Appendix 2: Temperature Measurements Across Batches

DAY	BATCH1	BATCH2	BATCH3	BATCH4	BATCH5
D6	30,04	31,96	31,56	31,47	29,94
D7	30,54	34,04	32,7	33,025	32,38
D8	31,12	33	33,5	33,5	32,78
D9	31,1	32,64	32,42	32,9	31,96
D10	31,24	33,34	35,12	33,85	32,32
D11	30,84	32,56	31,76	30,3	31,94
D12	30,66	32,54	31,96	31,55	30,48
D13	32,24	31,54	33,74	31,95	32,34
D14	30,16	32,36	31,52	31,58	32
D15	31,22	33	31,32	31,03	30,84
D16	30,54	30,58	31,66	31,17	31,28
D17	29,74	31,66	31,38	31,2	31,42
D18	31,04	33,12	33	31,42	31,4
D19	31,86	33,08	32,46	25,54	32,06
D20	30,36	31,6	32,58	31,65	31,06
D21	30,5	31,84	32,54	31,46	31,12

VI.3. Appendix 3: Composition and Preparation of Culture Media

VI.3.1. MRS Medium (Non-selective, enriched medium for lactic acid bacteria)

- Peptone: 10.0 g
- Meatextract: 8.0 g
- Yeastextract: 4.0 g
- Glucose: 20.0 g
- Sodium acetatetrihydrate: 5.0 g
- Ammonium citrate: 2.0 g
- Tween 80: 1.0 ml
- Dipotassiumhydrogen phosphate (K_2 HPO_4): 2.0 g
- Magnesium sulfate ($MgSO_4$): 0.2 g
- Manganese sulfate ($MnSO_4$): 0.05 g

- Agar-Agar: 18.0 g
- Distilledwater: 1000 ml
- **pH**: 6.2
- **Sterilization**: Autoclaved at 120°C for 20 minutes

VI.3.2. LB Medium (Luria-Bertani) (Non-selective, general-purpose medium for bacterial growth)

- Peptone: 10.0 g
- Yeastextract: 5.0 g
- Sodium chloride (NaCl): 10.0 g
- **Volume**: per 1 liter of medium
- **Sterilization**: Autoclaved at 120°C for 20 minutes

VI.3.3. Chapman Medium (Selective medium for Staphylococcus spp.)

- Peptone: 10.0 g
- Meatextract: 1.0 g
- Sodium chloride: 75.0 g
- Mannitol: 10.0 g
- Phenolred: 0.025 g
- Agar: 15.0 g
- **pH**: 7.4
- **Volume**: per 1 liter of medium
- **Sterilization**: Autoclaved at 120°C for 20 minutes

VI.3.4. Nutrient Agar (GN) (Non-selective, general-purpose medium)

- Meatextract: 1.0 g/l
- Yeastextract: 2.5 g/l
- Peptone: 5.0 g/l
- Sodium chloride: 5.0 g/l
- Agar: 15.0 g/l
- **pH**: 7.0
- **Preparation**: 28 g per liter
- **Sterilization**:Autoclaved

VI.3.5. SS Medium (Salmonella-Shigella Agar) *(Selective medium for Salmonella* and *Shigella* spp.)

- Peptone: 10.0 g
- Beefextract: 5.0 g
- Lactose: 10.0 g
- Sodium citrate: 8.5 g
- Bile salts: 5.0 g
- Sodium thiosulfate: 5.0 g
- Ferriccitrate: 1.0 g
- Neutral red: 0.025 g
- Brilliantgreen: 0.00033 g
- Agar: 15.0 g
- **Volume**: per 1 liter of medium
- **pH**: 7.0 ± 0.2
- **Sterilization**: Autoclaved at 121°C for 15 minutes

VI.4. Appendix 4:Enumeration of FecalMicroflora

Table 1: Enumeration of Fecal Flora on VRBL Agar

Colonne1	Colonne2	Colonne3	Colonne4	Colonne5
VRBL 10-3				
	J1	J7	J17	J21
Batch 1	1,98E+06	2,30E+06	1,59E+06	2,10E+06
Batch 2	2,14E+06	1,96E+06	1,89E+06	1,92E+06
Batch 3	1,88E+06	2,05E+06	7,10E+05	8,80E+05
Batch 4	1,76E+06	6,10E+05	7,50E+05	6,90E+05
Batch 5	1,93E+06	9,90E+05	9,10E+05	9,10E+05

Table 2: Enumeration of Fecal Flora on MRS Agar

Colonne1	Colonne2	Colonne3	Colonne4	Colonne5
MRS 10-3				
facteur de dilution	0,001			
	J1	J7	J17	J21
Batch 1	7,50E+04	3,00E+04	3,20E+04	2,50E+04
Batch 2	6,30E+04	3,10E+04	2,80E+04	2,10E+04
Batch 3	8,10E+04	2,86E+05	2,66E+05	2,56E+05
Batch 4	6,90E+04	1,70E+05	1,98E+05	1,42E+05
Batch 5	7,40E+04	3,00E+05	2,87E+05	2,87E+05

Table 3: Enumeration of Fecal Flora on SS Agar

Colonne1	Colonne2	Colonne3	Colonne4	Colonne5
SS 10-3				
	J1	J7	J17	J21
Batch 1	6,80E+05	7,20E+05	8,00E+05	4,00E+05
Batch 2	5,30E+05	6,90E+05	9,20E+05	6,60E+05
Batch 3	4,90E+05	4,50E+05	4,20E+05	5,00E+05
Batch 4	4,10E+05	3,70E+05	3,40E+05	4,00E+05
Batch 5	5,90E+05	3,40E+05	3,00E+05	3,90E+05

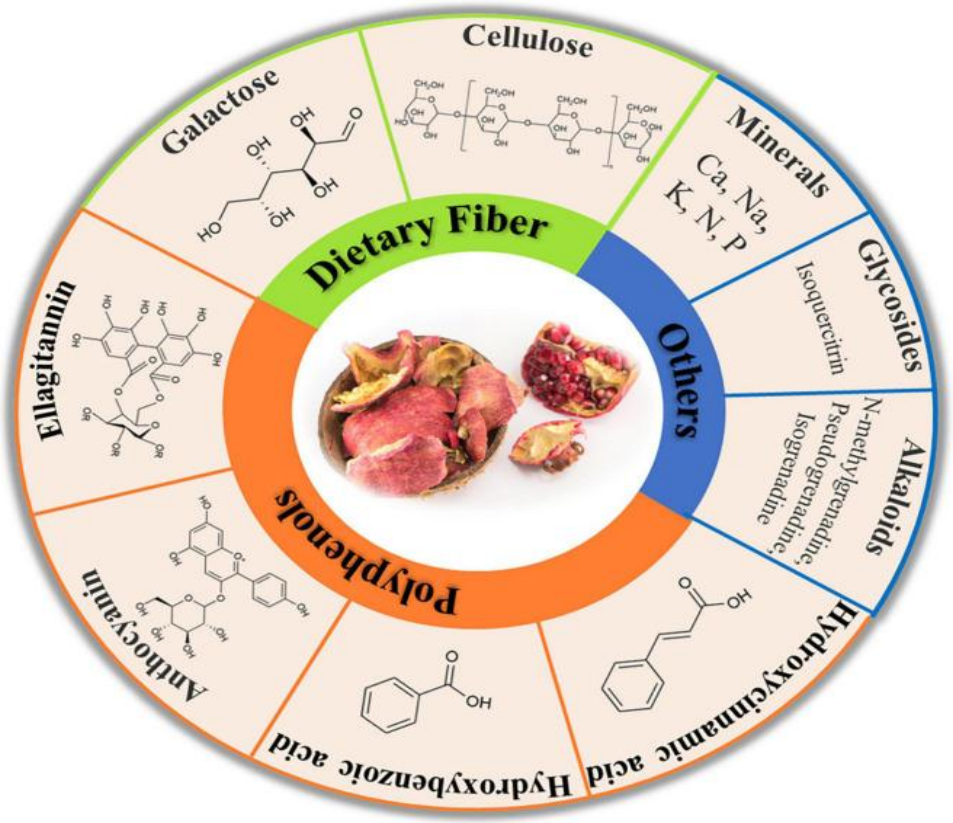
Table 4: Enumeration of Fecal Flora on Chapman Agar

Colonne1	Colonne2	Colonne3	Colonne4	Colonne5
CHapman 10-3				
	J1	J7	J17	J21
Batch 1	1,22E+06	2,78E+06	2,61E+06	2,63E+06
Batch 2	9,70E+05	2,39E+06	2,34E+06	1,81E+06
Batch 3	1,10E+06	2,18E+06	8,40E+05	9,60E+05
Batch 4	1,05E+06	1,93E+06	8,70E+05	8,90E+05
Batch 5	1,17E+06	1,99E+06	7,20E+05	7,90E+05

Table 5: Enumeration of Fecal Flora on Nutrient Agar (GN)

Colonne1	Colonne2	Colonne3	Colonne4	Colonne5
GN 10-3				
facteur de dilution	0,0001			
	J1	J7	J17	J21
Batch 1	2,45E+06	2,27E+06	2,25E+06	1,98E+06
Batch 2	2,31E+06	2,15E+06	2,13E+06	1,46E+06
Batch 3	2,59E+06	1,80E+06	1,95E+06	1,30E+06
Batch 4	2,46E+06	1,58E+06	1,72E+06	1,23E+06
Batch 5	2,38E+06	1,51E+06	1,48E+06	1,05E+06

VI.5. Appendix 5: Major Bioactive Constituents Identified in Pomegranate Peels



VI.6. Appendix 6: Examples of the Most Commonly Used Prebiotics