Study on Microbial Communities Modulation to Mitigate Obesity Related Gut Microbiota Dysbiosis

Submitted
By
Muhammad Nadeem Khan

Department of Microbiology
Faculty of Biological Sciences
Quaid-I-Azam University
Islamabad
2023

Study on Microbial Communities Modulation to Mitigate Obesity Related Gut Microbiota Dysbiosis

A thesis submitted in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

In

Microbiology

Submitted
By
Muhammad Nadeem Khan

Department of Microbiology
Faculty of Biological Sciences
Quaid-I-Azam University
Islamabad
2023

Bismillah

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

IN THE NAME OF ALLAH

Dedication

I DEDICATE THIS THESIS TO MY BABA JAN

AND TO MY ENTIRE FAMILY

Author's Declaration

I **Mr. Muhammad Nadeem Khan** hereby state that my Ph.D. thesis titled **"Study on Microbial Communities Modulation to Mitigate Obesity Related Gut Microbiota Dysbiosis"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

M. Nad

Mr. Muhammad Nadeem Khan
Date: 30-03-2023

Plagiarism Undertaking

"Study on Microbial Communities Modulation to Mitigate Obesity Related Gut Microbiota Dysbiosis" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: _____

Name: **Mr. Muhammad Nadeem Khan**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Study on Microbial Communities Modulation to Mitigate Obesity Related Gut Microbiota Dysbiosis"** was conducted by **Mr. Muhammad Nadeem Khan** under the supervision of **Prof. Dr. Muhammad Imran.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Mr. Muhammad Nadeem Khan** Signature: M.N90

Examination Committee:

a) External Examiner 1: Signature:

Prof. Dr. Ghazala Kaukab Raja
Director University Institute of Biochemistry & Biotechnology
PMAS Arid Agriculture University, Murree Road
Rawalpindi

b) External Examiner 2: Signature:

Dr. Allah Nawaz
House No. 667, Street No. 18
Sector I-8/2, Islamabad

Supervisor Name: **Prof. Dr. Muhamad Imran** Signature:

Name of HOD: **Prof. Dr. Naeem Ali** Signature: 17.5.23

Panel of the foreign Examiners/Referees

The thesis has been evaluated by the following foreign examiners/referees.

1. Dr. Marion Dalmasso

Assistant Professor, Department of Microbiology, Université de Caen, Caen 14032, Normandie, France.

2. Prof. Dr. Diarmaid Hughes

Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala 582 751 23, Sweden

List of Contents

List of Tables

List of Figures

List of Acronym/abbreviations (alphabetically)

AMA	American Medical association
CMA	Canadian Medical association
COG	Cluster of Orthologous Groups
CRP	C reactive protein
eggNOG	evolutionary genealogy of genes: Non-supervised Orthologous Groups
FDA	Food and Drug Administration
FFA	Free fatty acids
FMP	Fermented Milk product
FMT	Fecal Material transplant
GF	Germ free
GIT	Gastrointestinal Tract
GLP	Glucagon like protein
GRAS	Generally Regarded as Safe
HDL	High density lipoprotein
IBD	Inflammatory Bowel Disease
LAB	Lactic acid bacteria
LDL	low density lipoprotein
LPS	Lipopolysaccharides
MSPC	Multi Strains probiotic microbial consortium
NCD	Non communicable Disease
OECD	Organization for Economic Cooperation and Development
OUT	Operational Taxonomic Unit
SCFA	Short chain fatty acids
T2D	Type 2 diabetes
TG	Triglycerides
TLR	Toll like receptor
UC	Ulcerative Colitis
WHO	Word Health Organization
WOF	World obesity federation

Acknowledgments

بِسْمِ ٱللَّهِ ٱلرَّحْمَـٰنِ ٱلرَّحِيمِ

In the name of Allah, the Most Merciful and Beneficent

First, praise is to ALLAH, the Almighty, and the greatest of all, on whom ultimately, we depend for sustenance and guidance. I would like to thank Almighty Allah for giving me the opportunity, determination, and strength to do my research. His continuous grace and mercy were with me throughout my life and ever more during my Ph.D. research. Second, Swalat and Salam are always dedicated to our beloved prophet Muhammad SAW, the last prophet and the prophet who had brought us from the darkness to the brightness.

It is easy to acknowledge, but almost impossible to realize for long that we are mirrors whose brightness, if we are bright, is wholly derived from the sun that shines upon us**.** My Ph.D. was the longest journey of vicissitudes; I met many people at many turns. Everyone has played their part in making me, I am grateful to everyone. I wish, however, to give my sincerest gratitude and appreciation to all persons until this thesis can be finished and published. I do not remember the names of all of them; also, it is impossible to write them all here. However, some names are safe on the shelves of my mind.

I would like to thank my supervisor, **Prof. Dr. Muhammad Imran**, for the patient guidance, encouragement, and advice he has provided throughout my time as his student. I have been fortunate to have a supervisor who cared much about my work and responded to my questions and queries so promptly. I would also like to thank the entire **Academic as well as Administrative Staff of the Microbiology Department**, Quaid-I-Azam University, who helped me during my stay here in QAU. The Ph.D. tenure has been a challenging time, people helped me technically, financially, and morally and they are to be acknowledged. I am thankful to **Noureen Fatima** for her assessment in DNA extraction, for being a guide and helping hand in the IRSIP journey. Thanks to **Dr. Ansar** for allowing me to use the Nano spectrophotometer for the quantification of DNA. Thanks to **Prof. Dr. Naeem Ali** and **Dr. Hasan** for allowing me to use every lab instrument. I appreciate **Saroosh Sahar Siddiqi** for being a proofreader, **Sidra Irshad Khan** "جہانگیر ترین" for financial support; **Asad Abbas** (ex-Lab attend) for giving me pick and drop services whenever I needed.

I am grateful to the **IRSIP**, **HEC**, Pakistan because of its financial support I have done all the advanced experiments what I could not do here in Pakistan. I am thankful to all the Microbiology and Fermentation Section members**, Department of Food Sciences, University of Copenhagen, Denmark.** I found everyone here supporting in all means. I am grateful to my mentor **Prof. Dr. Sandris Dennis Nielsen** who gave me an opportunity to work under his supervision. In a few words of thanks, I think it is necessary to write in favour of Rasmus Riemer Jakobsen, Xiathon Mio, Zhuqing Xie, Bashir Aideh and Denitsa Vladimirova Stefanova because these people make it possible for me as a stranger to work efficiently in a new unfamiliar environment.

To all my Food Lab Seniors and juniors, I know you hate saying, "you're welcome," so I will do you a favor and not say thank you, but I am feeling it on the inside. I claim that Laboratory for Microbial Food Safety and Nutrition was a second home for me. Like a family, each individual has helped in each way. For Dear and respected **Misbah Tasbassum**, you are the person, I always found by myside and the person I have seen

happy with my successes and sad with my failures, the person I spent maximum time with, in the research and academic environment. You corrected, commended, appreciated and helped me whenever what I needed. Thank you **Madeeha Gaohar,** because whenever I asked you for help, you never refused. Thank you **Iffat Tahira** (late) for the favors you have given me. Thank you **Tanveer Ahmad** for your serious but jolly company. Thank you **Zumara Younas, Shahid Zaman** and **Ifra Gaohri** for being a part of my company. Thank you, **Saima Khalid**, for being my referee and guide in the matters of scholarships and positions. Thank you, Hafiz Arabab Sikandar, for giving me exposures and heling me out in some defined tasks.

I cannot forget my juniors, like a family, everyone is unique in their way. I enjoyed every moment with you people when we were together, which later turned into sweet and blessing memories, a reason of smile and happiness. I would like to say special thanks to **Naghmana Andleeb** among the juniors, a helping hand of me in experimental as well as in personal tasks. Whenever asked to do something, She Always said *Labaik*. I am thankful to **Sana Baba** for her unconditioned help during the time I needed much. I show my humble gratitude to **Syeda Momna Batool Bukhari**, she helped me and facilitated me during much needed time (me being on bed rest) without getting annoyed. I cannot express my gratitude enough for her tireless efforts she made for helping me.

Special thanks to my friends of **"Butt-Te-Bhatti"**, triangle **Shafqat Ullah, Muhammad Adullah** and **Waheed Ahmed** who were therapy to my depressions, reason for my smile, solutions to my problems and courage for my worries. The debates, dinners, chitchats, gossips as well as advice, rides to the airport, general help and friendship has all greatly appreciated.

Taking a break from the daily hustle and bustle is essential for mind to relax, recharge and rejuvenate. Thanks to **Uzair Aslam** and **Muhammad Awais** they made my weekends full of adventure and enjoyment.

To my friends scattered around the globe, thank you for your thoughts, well-wishes/prayers, phone calls, emails, texts, and visits and being there whenever I needed a friend.

If I could express thanks in all the languages of the world, I would do it to show how much I appreciate my Brother **Muhammad Idrees** for a mean of all kind support. Words cannot express my gratitude for **Mumma** Noor Khashi and **BaBa** Naseer Khan for being always my will wishers. I am thankful to all my siblings but specially **Shafia BiBi** the energetic soul and best friend ever.

Finally, I think it is important to remember my Fiancée **Habiba Noor** in the long story of gratitude. Even though you came into my life very late, you were overwhelmed as soon as you came. You had been a source of courage and smile for me.

Muhammad Nadeem Khan

Abstract

In the last few decades, obesity has become a global health problem affecting adults, teenagers, and children of both genders and has been linked with chronic metabolic diseases. The intestinal microbiota has appeared as a potent and vital endogenic feature that affects energy homeostasis and human health. The idea of gut microbiota dysbiosis mitigation has given a new insight into the treatment of obesity and metabolic diseases. Many options are used to modulate intestinal dysbiosis microbiota, among which probiotics are the most used option. However, it is reported that a single probiotic candidate cannot modulate the complex microbial communities of the gut. In this context, a multi-strains probiotic community developed from individual strains was used for mitigation of obesity-related gut microbiota dysbiosis in simulated *CoMiniGut* model. Initially, 143 bacterial isolates from 37 Dahi (a traditional fermented milk product of Pakistan) samples were chosen for microscopic, macroscopic, and phenotypic characterization. This returned 112 Gram-positive, immotile, catalase-oxidase negative candidates. Which further were evaluated for acid production, lactose fermentation, and curd formation. Seventy isolates were able to produce acids and utilize lactose. Their identification was confirmed through Rep-Seq, which were then exposed to low pH and high bile salts. Of these, 49 isolates survived, were evaluated for *in vitro* cell surface hydrophobicity, auto-aggregation, and survival *in vitro* simulated gastrointestinal fluids. All the 49 isolates presented *in vitro* cell surface hydrophobicity and auto-aggregation above 50% but only 22 isolates survived in the *in vitro* simulated gastrointestinal fluids. These isolated were evaluated for antagonistic potential against some common foodborne pathogens (*Bacillus subtilis* ATCC 19659, *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 27870, *Pseudomonas aeruginosa* ATCC 15422, *Staphylococcus aureus* ATCC 6538, *Streptococcus pneumoniae* ATCC 49619) and fungi (*Geotrichum candidum* QAUGC01, *Aspergillus flavus* QAUAF01 and *Candida albican* ATTC 90028), antibiotic susceptibility, anti-oxidative and *in vitro* cholesterol assimilation abilities. The isolates exhibited good antibacterial and antifungal activities and had *in vitro* cholesterol assimilation activity of 50–99%. These isolates were assessed for hemolytic, deoxyribonuclease, and decarboxylase activities and found negative for the tested characteristics. Two multi-strains communities were prepared from the isolates, one was consisting of 49 strains that survived in acids, bile salts and presented the cell surface hydrophobicity and autoaggregation and was named as multi strains probiotic community 1 (MSPC-1). The second community consisted of 22 strains that along with survival in acids and

bile survived well in the simulated gastrointestinal fluids and was named as multi strains probiotic community 2 (MSPC-2). These communities were evaluated and compared for *in vitro* synergistic growth, cell surface hydrophobicity, auto-aggregation, antibacterial activity, and cholesterol assimilation. In comparison to MSPC-1 MSPC-2 presented significantly high growth, high *in vitro* cell surface hydrophobicity (98%), auto-aggregation (83%), antipathogenic activity, and *in vitro* cholesterol assimilation (88%). The MSPC-1 was excluded from further characterization while survival of MSPC-2 was evaluated in the small intestine (TSI). The MSPC-2 survived the harsh conditions of the small intestine. Whole genomes of all the MSPC-2 isolates were sequenced and analyzed. The size of the genomes ranged from 1.8 to 2.4 Mb, GC contents were between 31 and 52%. On genome mining, it was found that none of the strains is extreme-drug resistant, bacteriocin genes were found in each genome, with no virulence and pathogenic genes. CRISPR genes were also present, indicating the immunity of the isolates to transfer elements. The MSPC-2 was used for the modulation of obesity-related gut microbiota obtained from Pakistani-origin obese volunteers (n=3) using *CoMiniGut.* It was observed that the probiotic community improved the bacterial count and richness of the experimental group. Alpha diversity was observed to be shifted from 0.7 to 0.8 and from 0.6 to 0.7 respectively when measured through Shannon and Simpson indexes. Beneficial bacterial genera were increased i.e., *Bifidobacterium* increased from 3% to 16.3%, *Coprococcus* increased from 0.79 to 1.34%, *Lactobacillus* from 5.38% to 13.71%, and *Prevotella* from 1.73 to 903%. Some bacterial genera, that contain mostly pathogenic members were decreased in response to probiotic treatment i.e., *Actinomyces* decreased from 7.34% to 2.23%, *Anaerostipes* decreased from 2.70% to 1.61%, *Clostridium* decreased from 9.68% to 7.47%, *Dailister* from 7.75% to 0.89%, *Enterobacter* from 5.15% to 0.08%, *Escherichia* from 1.07% to 0.01% and *Sutterella* from 2.31% to 0.12%. Furthermore, looking at the functionality, genes responsible for carbohydrate transport and metabolism, nucleotide transport and metabolism, replication, recombination and repair, translation, ribosomal structure, and biogenesis were increased for obese microbiota in response to probiotic treatment. Among the microbial metabolites, butyrate was specifically improved in both the groups with probiotic treatment.

The MSPC-2 can be used as an option for modulation of dysbiosis gut microbiota, after evaluation in the *in-vivo* trails.

Chapter 1
Introduction

1. Introduction

Rise in the incidence of overweight and obesity; accumulation of fats in the body based on BMI ≥30 m^2 (WHO, 2017) is a significant concern worldwide. It has been pandemic for the last twenty years and has affected people of all ages from both genders around the globe (Collaborators, 2017). According to WHO health statistics, Nauru is the number one obese country globally, with 61% of the population being obese. According to the same data, 36% of the US adult population is obese, 36% in Canada, and 26 % in the United Kingdom. In comparison, 8% of the adult Pakistani population are obese. According to WHO Pakistan health statistics, among the obese population, forty-one percent are males, and fifty-nine percent are females, and it is expected that this figure will double, quadruple, and even more in the following decades (WHO, 2018). Obesity is considered mother of all metabolic and chronic diseases and is linked with cancers, diabetes, hypertension, and heart diseases leading to a high rate of mortality (Tappia & Defries, 2020). The pathophysiology of obesity is very complex due to many combined factors such as behavioural factors, environmental factors, and genetic factors (Tappia & Defries, 2020). Nevertheless, the ultimate cause of all the factors is considered to be the imbalanced amount of energy i.e., the difference between consumed energy and utilized energy for basal metabolism or physical activities, resulting in fats accumulation and weight gain in the body (Jehan *et al.*, 2020). Overeating or eating nutritionally poor but dense food and diets with reduced physical activities result in energy imbalance (Romieu *et al.*, 2017).

It has been reported that the human intestinal tract harbors a large bacterial population ranging from 600-1000 species that carry hundred-fold more genes than the entire human genome (Guinane & Cotter, 2013). Over the last few decades, the role of gut microbiota in health and disease has been extensively studied, and it has been concluded that balanced gut microbiota is associated with health, and imbalanced gut microbiota (known as gut microbiota dysbiosis) is associated with diseases (Aoun *et al.*, 2020). The microbial profile of the human gut is unique, like a fingerprint for everyone, and has to act as an "endocrine" tissue or the "second brain" for each individual. As a result of growth and metabolism, intestinal bacteria produce a variety of Short Chain Fatty Acids and many other products that act as energy sources for the host and bacterial species (Kasubuchi *et al.*, 2015). The

SCFAs are also known as key regulators and contributors for numerous microbiological and host activities (Kasubuchi *et al.*, 2015). Associated with the structural and functional composition of intestinal microbiota, the SCFA profile is also linked with the health and disease condition of the host (Kasubuchi *et al.*, 2015). Such as the balanced gut microbiota have a balanced profile of SCFA while dysbiosis gut microbiota presents an imbalanced SCFA profile leading to altered metabolism (Kasubuchi *et al.*, 2015). Research has been focused on the modulation of gut microbiota for the treatment and management of obesity and associated metabolic diseases (Li *et al.*, 2017). Beneficial modulation of the gut microbiota is carried out with various strategies like using antibiotics, prebiotics, probiotics, and fecal microbiota transplantation (FMT) (Erejuwa *et al*., 2014; Jayasinghe *et al*., 2016). The use of probiotics (beneficial microbes) is safe and non-invasive method for modulation of gut microbiota. The known probiotics used include most members of the genera *Lactobacilli* and *Bifidiobacteria* because they have been classified as GRAS by FDA (Borriello *et al.*, 2003). Other probiotics they are being investigated include the members of *Bacillus, Lactococcus, Propionibacterium,* and *Enterococcus*. Numerous studies have stated that certain probiotic strains could decrease body weight and metabolic disorders, such as metabolic endotoxemia and insulin resistance. In human trials using stains of *L. rhamnosus* GG, *L. gasseri* BNR17*, L. gasseri* SBT2055*, B. breve* B-3*, L. casei* DN 114001, *B*. *lactis* BB-12, and *B*. *adolescentis* IVS-1 alone or in symbiotic form has proven their anti-obesity effects via specie and strain-specific mechanism in terms of satiety increase, body weight reduction or gut microbiota modulation (Barathikannan *et al.*, 2019; Wiciński *et al.*, 2020). Although experiments have been successful, it seems naturally impossible to modulate a community of hundreds of species of microbes using a single species of microbe. Hence, several studies have investigated the effects of multi-strain probiotics to determine their combinatorial efficacy compared to single-strain alternatives. For example, a combination of three probiotic strains (*S. thermophilus, L. bulgaricus*, and *B. lactis* Bb-12) resulted in weight loss in obese individuals after a 10 weeks trial (Mohammadi-Sartang *et al.*, 2018). In the same way, a preparations of nine probiotic strains, *B. bifidum* W23, *L. salivarius* W24, *L. acidophilus* W37, *B. lactis* W51, *B. lactis* W52, *L. casei* W56, *L. brevis* W63, *L. lactis* W19, and *L. lactis* W58 administration in postmenopausal obese women for 12 weeks resulted in glucose,

lipopolysaccharides, total cholesterol, and insulin reduction along with decrease in waist fringe and fats (Szulińska *et al.*, 2018). Similarly, a mixture of eleven strains comprising of *B. bifidum* SGB02, *B. animalis* subsp. *lactis* SGB06, *S. thermophilus* SGSt01, *L. plantarum* SGL07, *L. delbrueckii spp. bulgaricus* DSM 20081, *L. reuteri* SGL01, *L. acidophilus* SGL11, and *L. lactis subsp. lactis* SGLC01 was given orally to overweight and obese individuals for three weeks, resulting in significant body weight and subcutaneous fats reduction (Lorenzo *et al.*, 2017).

In the pre-industrial era, humans used to ferment milk to preserve its nutritional values, which has been a permanent part of the human diet since ever. The safety and health benefits of fermented milk products are very popular with the general public and have been used to treat many gastrointestinal diseases (Khorshidian *et al.*, 2020). The safety and therapeutic values of fermented milk products are associated with their microbiota, mainly lactic acid bacteria (Khorshidian *et al.*, 2020). The diverse lactic acid bacterial community present in fermented milk products are living together in a mutualistic way to produce a variety of metabolites like alcohols, aldehydes, acids, esters, Sulphur, and bacteriocin that give the product flavor and aroma, as well as protects it from bacterial and fungal spoilage (Bintsis, 2018). Furthermore, the well-known health-associated characteristics of particular LAB have led to the addition of selected strains as probiotic cultures with various applications in the food industry (Bintsis, 2018). Such as the use of probiotic yogurt (containing mainly *Lactobacillus* and *Bifidobacterium*) can treat and prevent obesity and diabetes while fermented milk product (FMP) enhances the expression of enzymes involved in numerous metabolic pathways and promote health without changing the composition of gut microbiota (Ejtahed *et al*., 2012; McNulty *et al*., 2011). Chinese fermented tea (Pu-erh) containing mainly *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* also improved the cholesterol level, body weight and fats when used to modulate the gut microbiota of diet induced obese mice (Gao *et al*., 2018). As so far, isolated probiotics from fermented food products are used for the treatment of obesity, they have shown significant decrease in Body weight, Body Mass Index and Fat Mass (John *et al*., 2018).

Dahi is a traditional fermented milk product that after Milk is the second most used item in different regions of Pakistan. It is consumed regularly as a part of daily diet and is

also used for the preparation of other cookery objects and therapeutic purposes. Traditionally, Dahi is prepared by adding unknown mixed starter cultures or spoonful of leftover junk from the previous badge of Dahi to boiled or sub-pasteurized Milk (Mudgal & Prajapati, 2017). Dahi is comparable to yogurt in its colour and appearance but unique in taste, texture, microbiology, nutritional, and therapeutic values. It contains all the nutrients present in source Milk, excluding the changes triggered due to heat treatment of milk, bacterial growth, and fermentation processes (Nawaz *et al.*, 2016). The microbiology of the Dahi is highly varied and is dependent on different factors such as the atmosphere, utensils, and human hands. In addition to numerous species of lactic acid bacteria, Dahi contain lactose fermenting yeasts, coliforms and spore formers (Bhattarai *et al.*, 2013). Microorganisms in the Dahi increase the rate of free fatty acids by lipolysis of milk fat and produce conjugated linoleic acid by using internal linoleic acid, which can confer nutritional and therapeutic values (Mudgal & Prajapati, 2017). Dahi has proved to have an anti-allergic effect, anti-oxidative effect, anti-carcinogenic effect, anti-diabetic effect, anti-atherogenic effect, lactose intolerance, immune enhancement, antimicrobial effect, and anti-diarrheal effect. Based on these properties, it can be said that if bacteria are extracted from Dahi and characterized, it may have probiotic potentials (Nawaz *et al.*, 2019b) .

The perception of mitigating gut microbiota is a significant approach for researchers and has directed to the utilization of some crucial methods in sustaining gut health through use of probiotics (Hou *et al.*, 2020; Nogacka *et al.*, 2020; Sergeev *et al.*, 2020). But due to the integral confines in sampling from human's gut, ethical concerns, and inter and intra individual variations, efforts have been placed to expand some alternatives like *in vitro* and *ex-vivo* simulating models that mimic the human gastrointestinal tract. Although none of these models can replace human-based trails, these can act as the best tools for proving conceptual and theoretical studies before human or *in-vivo trails* (Campana *et al.*, 2017).

There is speculation that more bacterial strains working in a mutualistic and synergistic way will have comparatively high potency for modulation of gut microbiota. That is why researchers are looking for new multi-strains microbial combinations that can significantly modulate the gut microbiota. In this context, we have developed a multi-strain probiotic combination characterized probiotics isolated from an indigenous fermented mil

Dahi and have evaluated their effect on the gut microbiota associated with obesity using a simulated *CoMiniGut* intestinal model.

1.2 Aim

Main aim of the current work is to design a multi-strain probiotic microbial community for beneficial modulation of obesity-related human intestinal microbiota.

1.2 Objectives

- Isolation, identification, and characterization of probiotics bacteria from artisanal fermented milk product (Dahi)
- Selection, Functional and Genomic Characterization of Multi-Strains probiotic community
- Modulation of the obesity related Gut Microbiota dysbiosis with Multi-Strains probiotic community

Chapter 2
Review of Literature

2.1 Obesity: Pathogenesis and Global Epidemiology

2.1.1 Introduction

Chronic metabolic disorders like diabetes, cardiovascular diseases and cancers contribute to more than 70% of the premature deaths globally, hence representing the foremost reason of life losses and early debility (Harris, 2019). Obesity a key risk feature for metabolic and chronic diseases, linked with declined life span of a predictable 5-20 years decreased dependent on the severity of the situation and concurrent complications (Weiss & Gepstein, 2019). The WHO described obesity as an unnecessary fat accumulation in the body that might harm the wellbeing of individuals and is measured as a BMI ≥30 kg/m^2. Obesity significantly intensifies risk of some metabolic disorders like cardiovascular, musculoskeletal, Alzheimer disease, depression, and some cancers. Obesity ruins the quality of life, causes unemployment, decrease individual's productivity as well as social gatherings (Dar *et al.*, 2018). Evidently, World Obesity Federation (WOF), American Medical Association (AMA), Canadian Medical Associations (CMA) and some other organizations, argued obesity an going and long-lasting disease evidently different from being just an increasing factor for other disorders (Rosen, 2014). Eliminating or at least decreasing the harms of obesity to health and humanities is the foremost priority of WHO, which let in the aim to stop obesity occurrence at a degree it was back in year 2010 among the prime objectives of 'Global Action Plan for the Prevention and Control of Non-communicable Diseases 2013–2020' (WHO, 2013). Currently, imbalance between taken and consumed calories is supposed to be main cause of obesity. But, reducing the intake and blowing up the consumption of calories, for decreasing weight, and reducing obesity are still ineffective (Teixeira & Marques, 2017). This failure could link with the complex pathophysiology and regional variations in the prevalence of the obesity. Coordination in different spheres like health, environmental, food processing and marketing, agriculture, transport and education is needed for management and reducing the obesity (Swinburn *et al.*, 2011). Therefore, an attempt has been made to highlight the deviation in the prevalence of obesity and regional variations related its pathogenesis.

2.1.2 Global epidemiology of obesity

The history of obesity is considered as old as the history of food is itself and has been reasoned to be consequence of evolution. Human bodily fat seems to have served a natural integral way for storing food assets. During ancient times, when famine was the only change of survival, natural selection rewarded the "prudent" genotypes of those who could store an incredible amount of fat and then could release it as economically as possible over the long term (Eknoyan, 2006). From the times when people were dying from famine till five decades back, being overweight was a sign of health, wealth, and beauty in many cultures around the globe. Over the past three decades, obesity spread around and affected the globe, then defined and declared a pandemic disease (Collaborators, 2017). In nations where the fat stature is considered a sign of beauty, obesity might occur earlier than in countries where society accepts small body size (Oreffice & Quintana-Domeque, 2016; Sides-Moore & Tochkov, 2011). Researchers of the NCD, Risk Factor Collaboration, have presented extensive data aiming at what means the obesity has transformed globally in the last four to five decades (Abarca-Gómez *et al.*, 2017). Based on the values of BMI collected from 129 million children, youngsters, and adults it has been concluded that the occurrence of obesity has risen in every country of the globe, and the number of obese people exceeds those that underweight.

The rate of a BMI ≥30 kg/m² differs country-wise and lies in a range from 3.7% in Japan to 38.3% in the United States Figure 2.1 (OECD https://www.oecd.org**)**. The incidences of obesity have been risen to a concerning stage in children and adults from 0.7% to 5.6% in males and from 0.9% to 7.8% in females. A rapid increase in BMI has mainly observed in many South Asian, Southeast Asian, Caribbean, and southern Latin American countries. Variations between the BMI ranged from no change in the countries of Eastern Europe to significant changes (1kg/m^2 per ten years) in Latin American countries. The incidence of obesity in children is more than 30% among children in the Cook Islands, Nauru, and Palau, by the prominent rise in the past few decades (Abarca-Gómez *et al.*, 2017). In a -review, the BMI of 51505 subjects recorded from childhood to adulthood has been compared. It has been observed that most obese children gained weight from the age of three to six years. Moreover, 90% of the children overweight at the age of three turned obese in their adulthood (Geserick *et al.*, 2018). It has been observed that in the last 40 years (1975-

2014), the rate of obesity in males has been increased from 3.2% to 10.8% and from 6.4% to 14.9% in females (OCED). The BMI and obesity incidence pattern are varied among different countries concerning gradient of increases, dropping down, and rising episodes (OCED). Remarkably, the level of BMI rise remained slow since 2000 in developed and underdeveloped countries than in the past century (OCED) (Abarca-Gómez *et al.*, 2017). It is still unclear if a decrease in BMI is either the treatment response or a preventing response to obesity as a health concern.

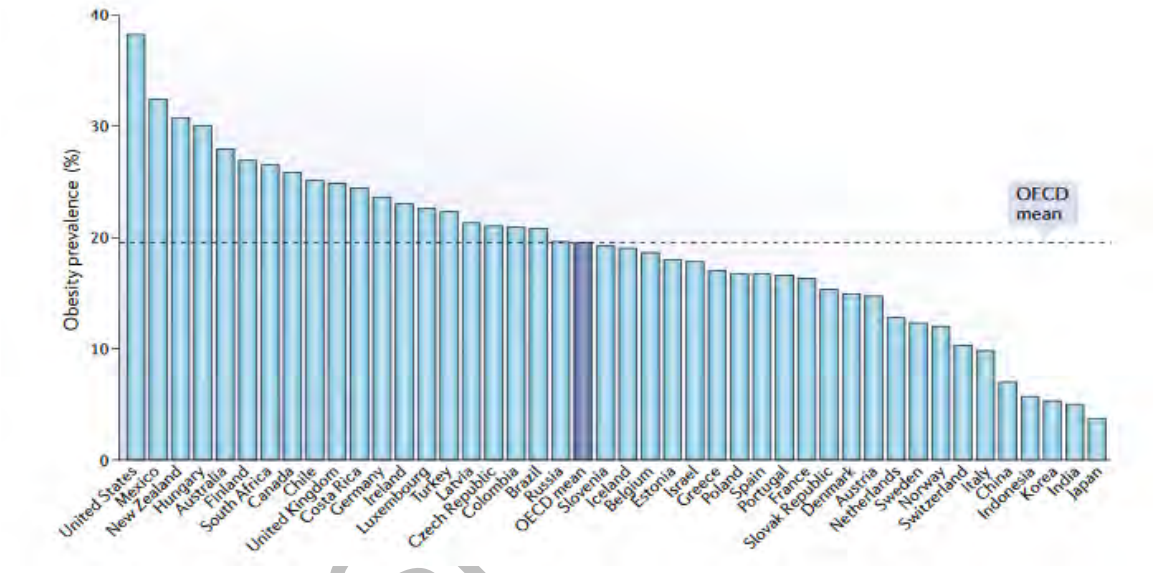

Figure 2. 1: Global incidence of obesity. Incidence of obesity (BMI ≥30 kg/m²) fluctuates among certain countries (Organization for Economic Cooperation and Development (OECD), 2018; percentage of adults with obesity from measured data). In 2015, through OECD countries, the mean frequency of obesity in adult subjects was recorded at 19.5% (dotted line) and laid in a range from <6% in Japan to >30% in the United States. Adapted from OECD

2.1.3 Regional differences in obesity

Differences in the degree and condition of obesity prevalence have been reported among different countries worldwide. Also, Individuals living in the same environment with similar economic statuses have been reported to be different in the state of obesity, reflecting an involvement of other factors. In the northwestern cities of Germany, obesity is 20% in the adult population, while in the Saxony Anhalt, it is more than 28%. Such differences might be associated with differences in individuals' socioeconomic statuses but can also be linked to other factors like physical inactivity and dietary patterns (Kim *et al.*,

2019). In the United States, obesity is less prevalent in its western part, while it is more prevalent in the southern part (Koh *et al.*, 2018). Along with socioeconomic status, other factors like ethnicity, poverty, unemployment, number of physicians in a certain area, fast foods, living set up, cultural norms, gaiety and a standard weight, and body figure accepted in the specific areas are linked to the incidence of obesity (Koh *et al.*, 2018). Differences in the economy of the same society might also have variations in obesity and related problems. Even inside a city, considerable local variances in obesity rates can happen, as revealed, for example, among neighbor hoods of the city Kiel situated in the northern Germany, obesity was highly dominant in neighbors with high rate of overweight and obese parents, obese/overweight siblings, parent's smoking, single parentage, low economy, reduced physical activities in male and high TV use in females (Lim *et al.*, 2017). Famous and known obesogenic arbiters like environment, fast foods, eating habits, transportation, and physical inactivity can have an inordinate effect on obesity in the regional and country milieu (Swinburn *et al.*, 2011).

In China, an abrupt shift from rural to urban life setup and automobile means of transportation could be among the fundamental causes of obesity epidemiology (Monda *et al.*, 2007). The prevalence of high obesity can be decreased in individuals shifting from a vicinity with high obesity and poverty to an improved economic area, aiming to explore further environmental or individual factors associated with obesity (Kim *et al.*, 2019). Similarly, the ethnographical research in two states with increased incidence of obesity, Nauru and Samoa, suggested that anti-obesity treatments cannot be blamed for unsuccessful due to body size norms of these countries (Hardin *et al.*, 2018).

The development of obesity was first noted in the 1970s in developed countries, then in middle-income countries, and finally in low-income regions. Which proposes that the development of obesity is the consequence of better economy and prosperity. Such as, in developing countries, signs of obesity emerge early in the adult population of urban areas who have higher socioeconomic status, while these signs also appear in the people of rural areas with an improved economy (Fox *et al.*, 2019). Disparately, in early 1999, childhood obesity appeared to decline or be stable in some developed economies like France, Norway, Denmark, Sweden, the United States, Japan, and Australia (Rokholm *et al.*, 2010). Though, differences in the pattern and state of obesity between or within the countries show not

only financial but also cultural, environmental, and other variances. The incidence of Obesity lies from less than 5% in regions like Bangladesh, Japan, Laos, and Vietnam, to more than 50% in countries like Nauru, Tonga, and Samoa, signifying the connections between genetical and ecological influences of an individual (Collaboration, 2016).

2.1.4 Pathogenesis of obesity

Obesity has complicated pathophysiology which is not yet fully understood. A combination of biotic and abiotic factors are the contributors to the pathogenesis of obesity. Biotic factor includes genetic, hormonal and Human Intestinal Microbiota while Abiotic factors contain environmental, behaver and dietary factors. Each of these factors is too diverse, comprising of many sub-factors. Further, these are interconnected either by direct or indirect means. The "Hot spot" in the pathogenesis of obesity is the Energy (Calories) imbalance, such as the difference between taken and expended energy (Figure 2.2). It has been stated that human and their forerunners had managed to survive in times of undernutrition when resources were limited. This social, environmental, and behavioral pressure during evolution makes them able to selectively inherit a genotype favoring overeating and low energy expenditure (Eknoyan, 2006). Humans who could bear extended starvation store and mobilize calories competently might have increased than those deprived of such adaptations, leading to over expression of genomic mutants that encourage the capacity to eat fast and store maximum calories (Yanovski, 2018). This further led to the anomaly of meals, incorrect frequency of eating windows, negligence of food's quality, and incorrect distribution of between specific groups of the diet. In early 1961, the average intake of calories per day per average person was 2300, which increased to 2800 in 1998, to 3000 in 2015, and is expected to be increasingly more by 2025 (WHO, 2018), Further accompanied with reduction in physical activity. That is promoted by the environment where we stay (house, office, working area, transport). The vibrancy of at least two-third of the Europeans is still unsatisfactory, but it is steadily declining. The WHO acclaims minimum physical activity for average weighted adult somewhat 30 min per day and for children 60 min per day. It is predicted that in Europe, most cars are used to cover a distance of 5 kilometers. Adding to such inconsequential explanations for weight gain, as stated above, it is also

vital to indicate such aspects as genetic, non-genetic biological, pharmacological, psychological, and environmental.

2.1.4.1 Biotic factors

I. Genetic: Many studies propose obesity as a genetic disorder, and the inheritance of BMI has been reported between 30 to 70%. Genetic obesity is either monogenetic reported in any of the genes encoding for leptin, leptin receptor, melanocortin four receptors or proopiomelanocortin or multi genetic where the mutation is reported in different genes at a time. It has been observed that alteration in single obesity-associated genes is a reason for obesity in a relatively small percentage of the population, while in most cases, the mutation in multiple genes responsible for food intake, metabolism, and energy hemostasis are linked with obesity prevalence (Wangensteen *et al.*, 2005). Some other genetic syndromes like Prader-Willi, Turner's, and von Gierki's are also associated with over accumulation of fats in the body (Gesta *et al.*, 2006).

II. Non-Genetic, neuro-hormonal imbalance: The non-genetic biotic factors which result in over accumulation of fats and body weight gain, include an imbalance in the endocrinal system, deficiency of growth hormones, and some syndromes like Caushing Syndrome and polycystic ovary syndrome.

i. Ghrelin: it is a neuropeptide hormone and is a key player in managing body fat, secreted in higher amounts by the fundus of the stomach and small amounts by the small intestine (initial section), hypothalamus, pituitary gland, and pancreas. From the stomach, it is directly secreted to the blood vessels and is circulated in the body. Receptors for the Ghrelin are present in the center never cells of the hypothalamus, a key regulator of hunger. The concentration of ghrelin rises at the time of hunger, particularly early to food intake, and decreases by food stimulus (Pradhan *et al.*, 2013). Its concentration also decreases when blood have high concentration of glucose or insulin. Moreover, it upsurges growth hormone ACTH, cortisol, adrenaline, and glucagon causes gluconeogenesis in the liver and constrains insulin secretion. It also stimulates lipogenesis in the adipocytes resulting in the increased uptake of glucose and triacylglycerols. At a threshold, ghrelin cuts adiponectin

production, decreasing the level of triacylglycerols and LDL and increasing HDL in the blood (Pradhan *et al.*, 2013).

ii. *Glucagon-like peptide-1 (GLP-1)* is a member of the colonic hormones, released by L-cells of the small intestine (final section) as a feedback agent to meal uptake. It raises glucose-dependent insulin release, holds stomach evacuation, stops glucagon secretion and hepatic glucose synthesis (Holst, 2007).

iii. *YY Peptide:* It is also an intestinal hormone secreted by the L-cells present in the jejunum, colon, and ileum when the meal is being taken. This hormone formerly releases the meal, even before food substances grasp the intestine, which possibly is associated with nervous stimuli. In later stages, hormone release is contingent on the nature of food material and its caloric value. The YY peptide reaches a high concentration if fatty food is taken, compared to proteins or carbohydrates containing meals of same caloric values. The concentration of YY peptide rises, approaching a high level to 1-2 hours when food is taken and stays at the same level for 6 hours. Enzymatic modification of the YY peptide happens during peripherical circulation, as it can cross blood-brain barrier, reach the hypothalamic arcuate, and have a vital part in managing and controlling food consumption (Le Roux & Bloom, 2005). During peripherical circulation, the YY peptide regulates gastrointestinal peristalsis by preventing the secretory role of the pancreas and stomach, delaying intestinal motility and gastric evacuating, which results in appetite reduction (Roux & Bloom, 2005).

iv. *Leptin:* it is a hormone released mostly by fats cells in the adipose tissues and by enterocytes of the small intestine at an extent relative to its weight (Myers *et al.*, 2008). It reduces appetite and speeds up gluconeogenesis and lipolysis in adipose tissue, increasing the concentration of free fatty acids in the bloodstream. Leptin also prevents insulin secretion and passage of glucose to adipocytes. In obese human subjects, high concentrations of leptin are noticed in the blood, but leptin receptors affinity is diminished, due to which obese subjects do not feel full despite taking high calories input (Münzberg *et al.*, 2005). The occurrence of metabolic problems of obesity in the form of mostly T2D or hypercholesterolemia is linked mainly with the expansion of visceral fats. Adipose tissues are thought to be necessary for the body's equanimity and are significant physiological organs. The basic role of adipocytes, which possibly is of more concern in

the pathophysiology of insulin resistance, is their capacity to accumulate lipids to form triglycerides (TG). Nevertheless, FFAs (free fatty acids) which cannot be deposited in adipose tissues in the final shape of TG, infuse into blood, then toward skeletal muscles and liver, increase gluconeogenesis and decrease the creatinine clearance. Too many FFAs, contrarily, prevent the deportment of glucose to the skeletal muscles, glucose phosphorylation, and its oxidation. Furthermore, in insulin resistance, adipose tissue macrophages being a cause of either pro and/or anti-inflammatory cytokines have a significant protagonist and the CRP (C-reactive protein), produced mainly in the liver, is a serum indicator of inflammation and tissue damage (Marnell *et al.*, 2005). It is thought that obese patients show a strong correlation between serum C-reactive protein and BMI, while a reduction in body weight leads to a decline in CRP level (Okita *et al.*, 2004).

III. Gut microbiota: obese people contain changed microbiota when compared to lean subjects' microbiota. It has been observed that obese people carry a higher concentration of *Firmicutes* and lower concentrations of *Bacteroidetes* (Silva *et al.*, 2021). Obesity-associated variances have also been identified in the shape of microorganisms, like *Clostridium innocuum, Eubacterium dolichum, Cateni-bacterium mitsuokai, Lactobacillus reuteri, Lactobacillus sakei*, and *Actinobacteria*, and a variety of the occasional Archaeaorganisms like *Methanobrevibacter smithii* (Silva *et al.*, 2021). This experimental indication proposes a strong connection between intestinal microbiota and body weight management, as is previously published in animal experiments. In 1980, Wostmann and his collogues investigated and found that germ free (GF) mice needed comparatively more energy intake to match and uphold the desired body weight that did the wild partner (Wostmann *et al.*, 1983). The results were later supported by Bached's group when they observed 47% greater total fats in the wild mice instead of germ-free mice taking higher calories. Moreover, when the intestine of germ-free mice was inoculated with the microbiota isolated from the cecum of wild mice, the body fat of GF mice was increased by 60% (Muscogiuri *et al.*, 2019). It has also been observed that GF mice, when given high carbohydrates and high fats, increased less in body weight than the wild partner did. While the GF mice did not develop any sort of imbalance in glucose digestion compared to the wild mice (Crovesy *et al.*, 2020). The research team then analyzed more than>5000

bacterial 16S rRNA sequences from the cecal material of both lean and genetically modified obese mice. They were able to observe variation in between two prominent bacterial phyla i.e., *Bacteroidetes* and *Firmicutes* of two types of mice. There were 50% less *Bacteroides* and 50% more *Firmicutes* in the cecal microbiota of lean mice when compared to the leptin mutated obese mice. Turnbaugh *et al*. then investigated the colonizing pattern of the microbiota of the GF wild-type mice with the microflora collected from the cecum of lean or obese donors; mice transplanted with cecal microbiota collected from obese mice showed a high percentage of body fats than did mice transferred with the cecal microbiota from lean mice (Turnbaugh *et al.*, 2006). Many researchers have explored possible mechanisms hiding the links between obesity and the microbiota that might act as a reason for the diverse obesity phenotypes observed with a variant microbiota profile, searching to evaluate how microbiota profile might control host energy regulation and if the persuaded modulations might be communicable (Crovesy *et al.*, 2020). It has been investigated that an increased number of intestinal *Bacteroidetes* members are directly linked with the fluctuated concentrations of SCFAs, especially propionate, butyrate, and acetate. These are the fatty acids, which binds to G-protein-coupled receptors (GPCRs), and precisely to the GPR41 (free fatty acid receptor 3, or FFAR3) expressed in the intestinal tract, adipose tissues, and the peripheral nervous system. This receptor attaches mainly to Gi/Go proteins, whereas GPR43 mostly stimulates Gi/Go proteins in the intestine and adipocytes. Moreover, data proposes that SCFA attaching to the GPR43 might mitigate the immunity by particularly dropping the production rate of inflammatory cytokines (Fernandes *et al.*, 2014), and by rising hypothalamic level of leptin sensitivity (Miranda *et al.*, 2019). Experimental trials on Gpr41-depleted mice recommend that stimulation of GPR41 by SCFA releases PYY, which promotes satiety and decreases diet ingestion; when released in the adipocytes, GPR41 increases the level of secretory leptin, resulting in the initiation of satiety (Miranda *et al.*, 2019). In the adipocytes, GPR43-pathway stimulation overturns insulin signaling, thus preventing fat deposition and promote lipolysis. Intestinal GPR43 triggers the glucagon-like peptide 1 (GLP-1) secretion, which then intensifies postprandial insulin production and reduces glucagon excretion. In line to support these experiments, Gpr43-depleted mice given high carbohydrates and high fats resulted in decreased fat accumulation compared to control wild lean mice. But, in contrast to lean,

obese individuals have a rich bacterial profile proficient in digesting carbohydrates, resulting in extra energy to their host, which then is accumulated in the form of lipid or glucose (Pakhomov *et al.*, 2020). In addition to these mentioned procedures, variations in the structure of the intestinal microbiota observed in obese phenotypes reduce the secretion of fasting-induced adipose factor (FIAF); subsequently, it is a circulatory lipoprotein lipase inhibitor (LPL), primes high rate of white fat accumulation (Muscogiuri *et al.*, 2019). Changes in the intestinal microbial profile to a high proportion of Gram-negative bacteria may also change the protein configuration in intestinal endothelium "tight junctions," resulting in elevated intestinal permeability, stimulating the captivation of microbial endotoxins like lipopolysaccharides (LPS) into the bloodstream via CD14 receptor requisite, that encourages amplified LPS-signaling cascades which results in the secretion of pro-inflammatory cytokines, as observed in the case of obesity and insulin resistance (Hersoug *et al.*, 2018). Fluctuations in the intestinal microbiota also trigger endo-cannabinoid system via stimulus of endothelium CB1 receptors , that pay to rise intestinal permeability, supplementarily elevating the plasma LPS concentration with augmented gut barrier interruption and improved endocannabinoid system movement in the intestine and adipocytes, exasperating the preliminary disorders and resulting in a spiteful sequence (Giovanna *et al.*, 2019).

IV. Pharmacological: Several medicines used repeatedly can also cause weight gain. Those include anti-depressants (amitriptyline, doxepin, mirtazapine, mianserin), anxiolytics, neuroleptics (phenothiazine derivatives, olanzapine, risperidone), anti-epileptics (valproic acid, carbamazepine), corticosteroids or insulin. The incidence of obesity is inseparably linked with fluctuations in the neuro-hormonal stability.

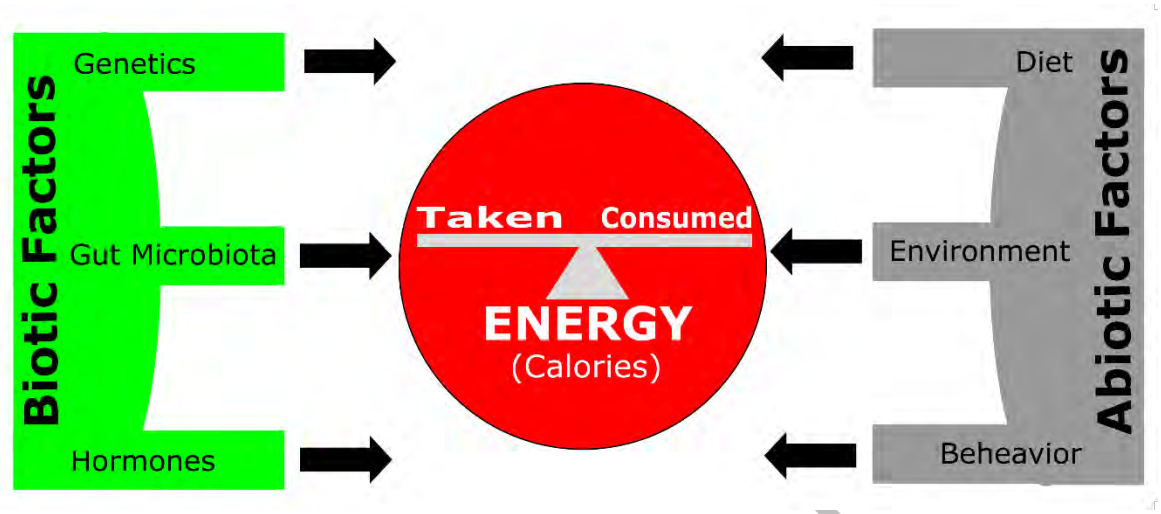

Figure 2. 2: Factors involved in the pathogenesis of obesity. The figure illustrates that Biotic and Abiotic factors play important roles in energy balance.

2.1.5 Obesity oversight as a disease

The apparent rise in the onset of extreme obesity also indicates the negligence of individuals and health care takers to diagnose and treat obesity at its initial stages. Obese individuals in many countries do not consider themselves obese and believe themselves healthy (Roberto & Kawachi, 2015). Different councils like AMA do not consider obesity as a disease. Such an approach often results in a delay of obesity treatment and lastly, a point where bariatric surgery is necessary (Bray *et al.,* 2017). Moreover, that is not a final and reliable solution to the global obesity incidence. Ignoring the spreading and chronic nature of obesity as a disorder subsidizes the continuation of this disease. When the body gains weight, it slowly leads to the physical inactivation of the body, mental dishonor, and a shame of the body figure. To manage and come out from this situation, readymade energy dense foods are taken which further leads to more weight gain (Stangl *et al.*, 2019). In few obese persons, stress and body weight disgrace leads to the addictive nature that leads to the spiteful weight gain cycle (Paterson *et al.*, 2019).

Additionally, health professionals have a negative attitude toward obese people, which renders a seriousness of patients towards treatment (Hong *et al.*, 2019). This ignorance has been rooted and originated at an elementary level; for example, obesity control and treatment have been included in the United States Medical Licensing Examination (USMILE) undergraduate medical examination (Kahan & Manson, 2019). The main issue

we are facing to treat obesity is the lack of basic and fruitful information of understanding the complexity of obesity (Flint, 2019). It's dire need of time that the complication of obesity must be simplified to some adoptable reasons that the general public, health professionals, and policy makers could easily understand.

2.1.6 Risk factors associated with obesity

Obesity is the consequences of various interconnected factors ranging from individuals' dietary habits, physical activities, energy utilization and his/her intestinal microbiota to the effect of environment and society, individuals are living in (Butland *et al.*, 2007). The circumspection 'Tackling Obesities' scheme described seven main and essential factors that initiate and progress obesity for individuals or the public (Butland *et al.*, 2007). These equally important factors for causing obesity are physiology, psychology, physical vigor, dietary consumption of an individual during food processing and production, psychology and physical activities are persistent in the living environment of that individual. According to the German Health Interview and Examination Survey for Children and Adolescents (KiGGS), striking menace players in childhood obesity are obese parents, poverty, shifting from rural to urban areas, and high birthweight (Beyerlein *et al.*, 2014). In another meta-study of more than eight thousand children in the United Kingdom, parental obesity, early overweightness, birthweight, sedentary routine, stunt growth, and insomnia are supposed main players in the onset of obesity (Reilly *et al.*, 2005). Though the significance of parent's obesity might propose the contribution of genetics, the association of genetics with obesity describes a small account of contribution in the onset of obesity. Therefore, it can be claimed that general obesity genomic deviations don't precisely develop obesity but might enhance the obesity associated risk under obesogenic situations (Kopelman, 2000). As two comparative studies confirmed, that introduction to positive and negative energy homeostasis causes variations in body weight than between identical twins (Bouchard *et al.*, 1990).

2.1.6.1 Manifestation to food commercials

Food industries display commercials for the advertisement of many obesogenic foods and beverages that are nutritionally poor and energy-dense with a high number of sugars and fats. In youngsters, preference for fast foods (energy-dense) rises through or soon after they

are introduced to the food ad (Sadeghirad *et al.*, 2016). It can be concluded that increased fast food consumption has an impact on the genotype of youngsters. As carriers of high risk single nucleotide polymorphism gene (FTO) responsible for the accumulation of fats and development of obesity is more reactive to the food advertisement as compared to normal counterparts (Gilbert-Diamond *et al.*, 2017). Genome-wide association studies have proven the FTO gene a most vital driver for change in BMI and onset of obesity (Loos & Yeo, 2014). The FTO risk allele carriers have declined sensitivity to satiety and increased energy intake, providing evidence on the importance of the brain, where FTO is expressed highly in response to food advertisements (Hess *et al.*, 2013). Some people cannot control their unconscious or sub-conscious stimuli to food associated signals due to the inflection of numerous neuro-physiological pathways (Cohen, 2008). Moreover, the central nervous especially dopamine, mesolimbic, opioid, endocannabinoid, and melanocortin regulate not only appetite and satiety but also thermogenesis and spontaneous functions hence efficiently guard the body weight (Richard, 2015). It can be suggested that regulation of individual food preferences by genome might make it hard to control obesity at the individual level. The unconscious and subconscious control of energy balance raise a question on the idea of aiming individual determination associated with diet and exercise options as obesity preclusion or treatment.

2.1.6.2 Fluctuating lifestyles

Today, we are living in progressively obesogenic atmospheres, which greatly affect conduct and life adoptions. The augmented occurrence of obesity during the past five decades has resulted in decreased home cookery, greater dependence on ready to eat food, increased use of air conditioning, decreased physical activity, automation of laborious works, excessive use of technology, snack consumption, convincing food advertisement, and other fluctuations (Löffler *et al.*, 2017). The food industry's objective is to achieve revenues and hence sponsors large servings. Due to this, regular snacking, stabilization of sweets, soft drinks, and fast food are becoming very popular and inescapable in our daily routines. The westernized lives are central to a drastic rise in the onset of obesity; it can be said that this type of environmental factor can cause obesity even in genetically similar individuals. Such as the incidence of obesity was lower in Indians who were shifted and

living in Mexico than those who were migrated and residing in USA (Schulz *et al.*, 2006). Similarly, native Nigerians had decreased BMI compared to those who had been shifted and settled in the United States (Rotimi *et al.*, 1995). Remarkably, the increase in the incidence of obesity seems augmented in developing countries where behavior and environment are changing fast. Like obesity incidence in a country (Jamaica) with average income increased quickly between the year 1995 and 2005 than a country (Nigeria) with low income and a country with high-income country (USA) (Durazo-Arvizu *et al.*, 2008). The substantial variation in obesity incidence among different countries proposes a robust impact of the environmental factors on how important contributors of the obesity epidemic mark cultures differently. But drastic shift in global dietary pattern and physical inactivity are widely accepted cause of obesity.

2.1.6.3 Role of the global food stock

The global food chain is also a significant contributor to the onset of obesity. For example, the pacific islands having four times more obesity than the entire world, is now the most obese nation on the globe (Collaboration, 2016). Various reasons have been suggested to highlight the vulnerability to increased obesity gain in these countries, including genetic tendency, topographical isolation and the inability to produce enough food for their use (Snowdon & Thow, 2013). The later reason related to food shortage might expose the residents to the international food chain and presentation than the nations which are self-sufficient in food production (Mclennan & Ulijaszek, 2015). Similarly, small and congested societies appear to be liable to social fluctuations and global food marketing (Mclennan & Ulijaszek, 2015). The Instance of the rapid obesity development in the Cook Islands describes that obesity might occur when rapid social fluctuations are made to the societies with increased interdependence and interconnectedness (Mclennan & Ulijaszek, 2015). Together with the studies that obesity incidence in Cuba reduced in the area of the economic crisis in early 1990, it proposes that obesity is not only an outcome of specific preferences and individuality (Mclennan & Ulijaszek, 2015).

2.1.6.4 The energy 'Tossing Point.'

Globally, but mainly in the prosperous countries, the mechanical insurgency of the previous century with automation, new sources of transport, and robotics resulted in a decline in hominid energy requirement (Swinburn *et al.*, 2011). Though these fluctuations had already been recorded at the start of the 1900s, a clear increase in obesity incidence happened only from the 1970s forwards (Swinburn *et al.*, 2009). Hence, it has been speculated that energy hemostasis in communities in developed countries is defined through an energy "tossing point". In developed countries along with the USA the tossing point happened in the era when the food market for refine fats and carbohydrates noticeably improved (1960 and 1970) (Carden & Carr, 2013) (Figure 2.3). Till 1960, reduced energy expenditure was balanced by reduced energy consumption, but afterward intake of the energy increased without expending the energy or decreasing intake of the energy (Swinburn *et al.*, 2011). Availability of inexpensive food has also hypothesized a reason for high energy intake and ultimately obesity prevalence (Hall *et al.*, 2009). Hypothetically, the physical and societal mechanisms paying to a constant reduction in energy requirement might also pay to a shift in a society's energy hemostasis. Alteration in the body fatty mass of children foreseen from improved United States food energy source amid 1970 and 2000 were same to the calculated specific weight gain through that time (Swinburn *et al.*, 2009). Furthermore, a survey conducted in the UK showed that high energy consumption might completely describe the detected rise in body weight, at minimum in females (Scarborough *et al.*, 2011). This finding favors the theory that rise in food stock is vital to describe an increase in normal BMI and high obesity incidence (Swinburn *et al.*, 2011). Generally, demonstrative dietary studies in the UK proposes that change in the normal body weigh in male between 1986 and 2000 might be due to both high energy consumption and decreased physical activities (Scarborough *et al.*, 2011). An analysis of 1399 adults suggested that high energy consumption is a key player for increasing BMI and hence the main contributor to rising obesity in the communities. The start of the 'weight-gain phase' (Figure 3) during the early 1970 might also be observed as a reply to the strategies adopted for the improvement of the food market. Like, the 1969 White House Conference on Food, Nutrition, and Health spoke while addressing starvation and malnourishment suggested the procedures and guidelines needed to tackle malnutrition (Mcginnis & Nestle, 1989).

Concerning the incidence of obesity, various countries, including US, now encountered the objectives of changing guidelines, agricultural amendments, food trade and other divisions in means that expand the food market in agreement with nutrition rules and to ensure healthy preferences more simply accessible (Mcginnis & Nestle, 1989). Similarly, consumption of sugared drinks has been increased globally, resulted in high energy intake and obesity occurrence (Schulze *et al.*, 2004). Moreover, genetic tendency to obesity in specific subjects have been linked with increased sugared drink uses, suggesting the role of gene to environment collaboration in development of the obesity (Qi *et al.*, 2012). Why is obesity not affecting everyone? Energy dense foods lacking fibers while saturated with sugars and fats are consumed widely. It can be expected that everyone must develop obesity in these circumstances. These deviations are the vital contributors to the onset of obesity, but in an environment ranging from a state to its city, area, or even to kinfolks, the normal body weight varies significantly (Risk, 2016). Physical, genetic, or behavioral aspects are assumed to be the central regulators or arbitrators of energy hemostasis. A few of the structure's causal the heterogeneous biotic action to common milieus are not exceptional to humans. For instance, a sub-strain of the C57BL/6 mice (commonly used as control mice) is impervious to obesity persuaded by an high fat diet (Heiker *et al.*, 2014). The inherent distinction in one a rare genetic locus might describe this inconstant turn to obesity tempting diet generates in this animal model (Heiker *et al.*, 2014). Obviously, in humans, the mediators might also contain culture-based or adopted behavior plus food takin frequencies set against snacking, diet patterns, choosing exercises or computational games, and other aspects. Adding to these behavior changes, biological aspects like insomnia, stress, depression, weight disgrace, and discernment might also be vital in the circumstances of weight gain. Some social norms might also engender epigenomic alterations to the onset of obesity, including aged women giving their first births, extended contact of childbearing females to the obesogens, and stress agents that might cause considerable genomic changes in predisposition to obesity in the progenies. Genomic aspects like BMI variation might have been ignored for the ensuing reasons: monogenic obesity reasons are often occasional and are insufficient to describe the obesity pandemic; a combination of various small obesity-associated loci present on the genome may cause the obesity phenotype; Genome wide associated studies have been tried to investigate some

erratic, but physiologically related, genetic deviations; and the part of epigenomic alterations has not been fully clarified so far (Panzeri & Pospisilik, 2018). Considerable BMI differences might be a cause of gene-environment or gene-behavior synergism, along with the uterine environment. Interestingly, a mother's diet in the period of gestation can change the degree and pattern of DNA methylation that can continue for the decades in descendants and might even be transferred to the descendants (Panzeri & Pospisilik, 2018). Chemical contaminants persisting in the environment along with the residues of some insecticides like N, N diethylmeta toluamide (DEET), phthalates, and dioxin can change genomic manipulations even more intensely and thus elucidate modifications in predisposition to obesity. Inherited epigenetic procedures might be the reason of the obesity pandemic. Obesity is supposed to be a di-model illness in which a TRIM28 (also called TIF1β) based system is accomplished of causing obesity in non-Mendelian, 'periodic' way. Despite inkling the fact that the prevailing obesity epidemic is an outcome of the change in BMI distribution arch to the right, the di-modal suggest that epigenetic changes might "cause" obesity in some individuals while the others may not affected (Dalgaard *et al.*, 2016; Quarta *et al.*, 2016). Another idea which describes variations in BMI among human population is that vulnerability variances are natively present in the management of food intake and that respond to overeating. Explaining the idea, the modulator of G protein signaling 4 (RGS4) gene is known to control food intake and respond to diet mediated obesity. The human RGS4 gene is linked to increased body mass and obesity pathotype, and a high concentration of striatal RGS4 protein could be found in overweight individuals (BMI ≥25 kg/m^2) (Michaelides *et al.*, 2018). Conclusively, we are still at the initial phase of knowing how to explain the state of susceptibility to obesity in obesogenic situations, but genetic and epigenomic factors have a crucial role and are yet to be fully explored.

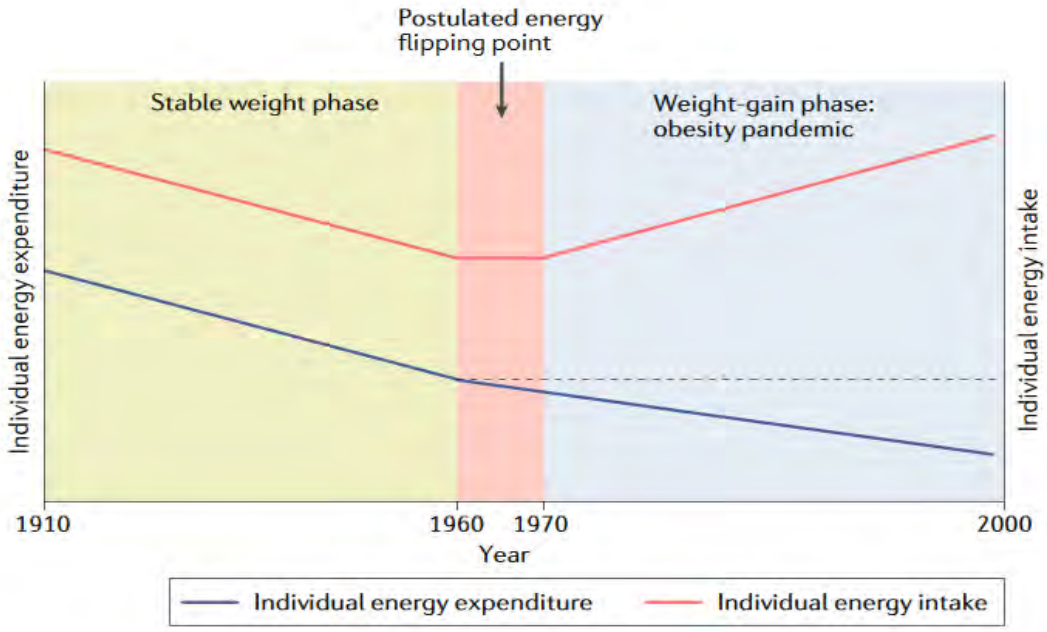

Figure 2. 3: Food consumption (red line) and energy expenditure (blue line) in the US in the period of 1910 to 2000. Energy expenditure abridged due to mechanical variations in the factory and increasing automation. This decrease in energy expenditure was corresponding to an equivalent decrease in energy intake and caused a constant weight segment. Owed to rise in making energy-dense, carbohydrate-rich and fat-rich food items in the US, in 1970s an energy 'Tossing point' characterize start of the weight-gain period, where amplified energy consumption was disproportionate to either plateauing (dashed line) or further falling energy expense requirements (exact measurements of energy expenses are unreachable)

2.6.7 Retrogressing and treating the obesity

The WHO defines some actions to avert obesity, including influencing the settings and societies in such so person's choice for healthy natural food and physical activity are the easiest, highly accessible, and reasonable. No country has been flourished in moving back from the existing pandemic state of obesity to the normal state so far. Somehow, Cuba proved that reappearance to general poverty, which. Began due to financial emergency in the mid 1990s and resulted in mean weight loss of ~5 kg, might significantly decrease obesity occurrence. In principle, intrusions must be designed to encourage behavioral modulations (like schooling, fitness preferment, societal advertising, and motivations for healthy living). Social and political activities like laws, regulations, and policies must be initiated to limit key causes of obesity. It might contain implementation of taxes on sugared

beverages, defining nutritional values for food servings at nurseries and/or play-group schools, and restricting the advertisement of unnatural foods for children (Weihrauch-Blüher *et al.*, 2019). The Chile and Mexico, governments, announced a sugared beverages tax in 2014, which was resulted in a significant reduction in the purchase of sweetened drinks, but health-related effect and reducing obesity is yet to be assessed (Bakhti *et al.*, 2019). Policies must be planned for food industries to prepare and process foods with low percentages of fats, sugars, and salts 7,10,43 so that an individual can efficiently bound him/herself for taking limited energy from the pool of energy dense foods, consuming healthy foods like fresh fruits, green vegetables, and legumes, and involving inconsistent physical activities. However, only a few countries around the globe have achieved the introduction of policies that led to solutions to limit occurrences of obesity, as government officers are unwilling to make laws limiting freedom of choice.

Moreover, food and associated industries are active against the anti-food marketing policies of the government (Mialon *et al.*, 2017). It is not possible to reverse obesity without the involvement of government headship. An organized and systematic approach is needed with several divisions engaged and with a primary population body weight data and treatment results precisely supervised and assessed.

Moreover, central databases empower checking the implementation, long time preservation, and availability of healthy food strategies; such databanks include the World Cancer Research Fund's NOURISHING site and the Global database on the Implementation of Nutrition Action (GINA) (Hawkes *et al.*, 2013). Numerous vital fences prevent developments in treating the obesity epidemic; the central one is nearly zero interest from the public for any political activity. Limited funds and subsidies, weak planning and the inexistence of devoted administrations pay to slow down improvements in dogmatic modifications (Popkin *et al.*, 2013).

2.2 Human Gut Microbiota: A Therapeutic Target to Combat Obesity and Associated Commodities

2.2.1 Introduction

Obesity is a chronic, reverting, complex neurobehavioral disease, where over-accumulation of body fats results in adverse metabolic, bio-mechanical, and psychosocial health consequences (Blüher, 2019). In the year 2013, obesity has been declared a pandemic disease that is affecting people of all ages and social groups of both the developed and underdeveloped countries and is typically linked to many metabolic disorders, including lactose intolerance, T2D, hypertension, dyslipidemia, fibrinolysis syndromes, cardiovascular problems and/or non-alcoholic fatty liver diseases along with some cancers (Philip & James, 2013; Upadhyay *et al.*, 2018). Since the last few decades, different causative reasons for obesity have been explored, like genetic, behavioral, and environmental factors (Bray *et al.,* 2017; Hales *et al.*, 2020). Numerous convincing scientific evidences favor the notion that obesity occurs in vulnerable individuals when exposed to obesogenic environmental or social factors (Lee *et al.*, 2019). The most frequent and accepted reason for the onset of obesity is a prolonged imbalance between taken and consumed energies in the form of calories (Romieu *et al.*, 2017).

In this regard, the human intestinal microbiota is crucial and has been the research target in current years. Normal and balanced gut microbiota have been associated with structural and biological intestinal structure that increase the absorptive area, regenerates the villi, rise the intraluminal fluid and quickens intestinal transit (Rosenbaum *et al.*, 2015). Intestinal microorganisms, which begin to accumulate from birth, interact with the immune system, help in immunity development, contribute to toxin and carcinogen demolition, and prevent pathogen's attachment to the intestinal linings (Bamola *et al.*, 2017). Further, human intestinal microbiota has potential metabolic activities, has a role in digestion and extracts energy through biochemical break down of diet contents, mining vital nutrients, vitamin biosynthesis, and enhancing the absorption of minerals (Rinninella *et al.*, 2019b). While, altered gut microbiota has been stated to favor the development of obesity due to appetite intensification, and after then quickens energy extrusion from the diet (Rinninella *et al.*, 2019b). The dysbiosis gut microbiota modulates substrates' development for repository fat biosynthesis (Venegas *et al.*, 2019). It makes compounds engrossed into the

circulatory system, which pay to the progress of obesity-associated problems, then they surge both tissue inflammatory impairment and insulin resistance (Zhang *et al.*, 2018). Based on these findings, it has been assumed that beneficial mitigation of the intestinal microbiota might offer an innovative curative goal in obesity.

This review aims to link human obesity to the intestinal microbiota with the help of existing literature that could help in the treatment or prevention strategies against obesity.

2.2.2 Gut microbiota: the globe of tinny creatures

Subsequently, when Ilya Mechnikov and Robert Koch were granted a Nobel prize in medicine and physiology in the 1900's for their work that connected microorganisms to human health. Then numerous factors related to host and microbe interaction, both contiguous (like oral cavities, intestinal surfaces, skin) and sequential (genetic and senescence), have been unraveled. Currently, revealing that the human's body harbors ten times more microorganisms than the human somatic cells (Savage, 1977), it is simply believable that humans and microorganisms have been inclined through constant friendly co-evolutionary past. The human gastrointestinal tract (GIT) carries a rich and diverse microbial community that accumulates more than 100 trillion microorganisms. The weight of microbial cells in the intestine has been predicted to be 10^{11}-10^{12} /milliliter, making the intestine one of the most compactly colonized bacterial niches identified on the earth. Furthermore, the microbiome code hundred-folds more genes than the entire human genome. This finding favors the notion that we are only 10% human and 90% microbes in terms of cells and 1% human and 99% microbes in terms of genes Figure 2.4. It is strongly believed that the gut microbiota, a "Mini Globe" within us, performs vital biologic and metabolic functionalities that cannot be achieved by the coded functional human genome (Ley *et al.*, 2008; Rowland *et al.*, 2018). This complicated synergistic mechanism and its expansion are probably hooked on the connections between the host, microorganisms, their genetics, and the environment (Luca *et al.*, 2018). Accurate and refined composition of the intestinal microbiota is still unknown; however, development in metagenomic technologies has recently initiated to dig out the structure of intestinal microbiota (Feng *et al.*, 2018). It has been proposed that each individual's intestinal tract harbors a unique and specific (fingerprint) cluster of 1000 to 1500 bacterial species representative of seven phyla, namely

Bacteroidetes, Furmicutes, Proteobacteria, Actinobacteria, Verrumicrobia, Acidobacteria, and *Fusobacteria* (Allen *et al.*, 2018). The intestinal tract carries both aerobic and anaerobic bacteria, but anaerobes outnumber aerobes by 1-2 logs (Bellali *et al.*, 2019). Two phyla *Bacteroidetes* and *Furmicutes* make 90% or above of the total bacterial population present in the gut of normal healthy adults. Being the dominant representative of the gut, *Bacteriodes* makes 65.4% of the bacterial population (Bamola *et al.*, 2017; Méndez-Salazar *et al.*, 2018; Shin *et al.*, 2015), that contain dominant genera of *Bacteriodes* and *Provetella* respectively. *Furmicutes* is the second most populous phylum with the percentage of 24.4 containing more than 200 genera like *Lactobacillus, Bacillus, Clostridium, Enterococcus and Rumincoccus.* The *Clostridium* genus represents 95% of this phylum (Rinninella *et al.*, 2019b; Seong *et al.*, 2018; Shin *et al.*, 2015). With members from various genera, *Proteobacteria* phylum constitutes 4.5% of the bacterial density within the human gut (Gupta, 2000; Rizzatti *et al.*, 2017; Shin *et al.*, 2015). The phylum *Actinobacteria* is less abundant having percentage of 2.2 and mainly represented by *Bifidobacterium* genus (Shin *et al.*, 2015). The remaining three phyla collectively makes 2.8% of intestinal bacterial population (Rinninella *et al.*, 2019a; Shin *et al.*, 2015) (Figure 2.4).

Significantly, due to sampling problems from different parts of the intestinal tract, studies exploring gut-associated microbiota's composition and functions have been conducted using fecal material (Raoult & Henrissat, 2014). Though the intestinal microbiota communities studied in the fecal material are not the accurate representatives of the intestinal tract, this cautious evidence to be and further supports the prerequisite of specific and accurate alternatives.

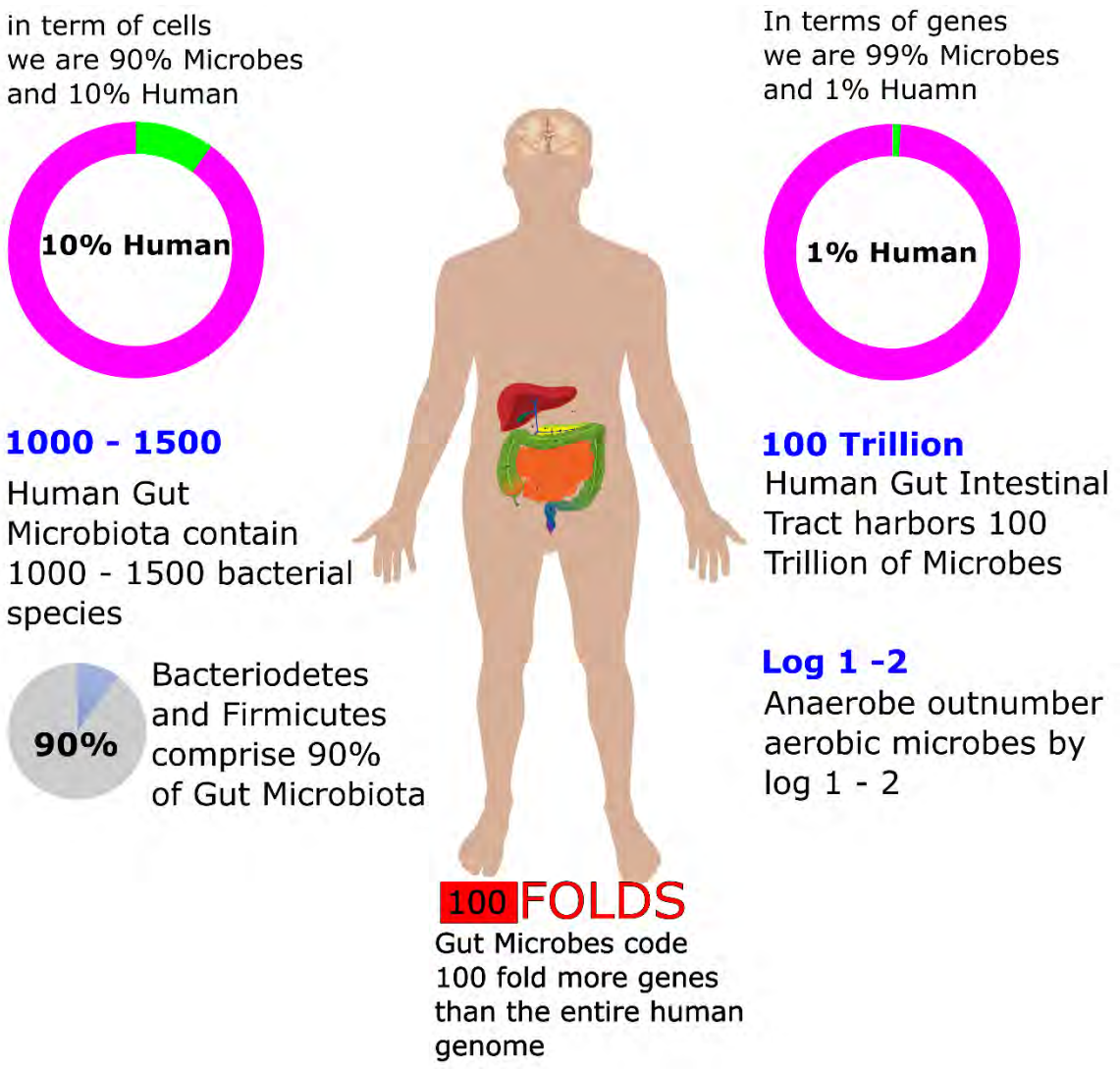

Figure 2. 4: Composition of Gut microbiota in terms of Numbers. Important numbers related to the Human Gut Microbiota such as number of bacterial cells, number of microorganisms, number of aerobic and anaerobic bacteria, percentage of important Phylum and genetic capability of the microbes is illustrated in the figure.

2.2.3 Gut microbiota and obesity development

2.2.3.1 Intestinal microbiota and energy balance

Unbalanced energy (calories) is proposed as a possible reason for the onset of obesity (Bäckhed *et al.*, 2004). In recent years, intestinal microbiota, an ecological and environmental factor involved in energy balance, is considerably investigated and correlated with metabolic functions and body weight management (Blaut, 2015). Despite some prominent differences between humans and rodents (excellently explored by

(Rosenbaum *et al.*, 2015), most studies have been done on rodents. Many mechanisms have been explained and suggested through which microbiota might affect energy balance. Such mechanisms are poorly explored in human subjects and must rely on the available information known about or proposed for energy homeostasis in humans. Body weight gain and loss present an imbalance between the join capacity of the energy intake and output. To persuade or endure changes in body weight, microbiota-associated mechanisms must someway interrupt this coupling. In this context, starting from food intake, some eating habits like mindless eating (Wansink, 2011), emotional eating (Adriaanse *et al.*, 2011), stress-eating (Adam *et al.*, 2007), skipping meals (Karatzi *et al.*, 2017), dieting (Polivy & Herman, 1999) and eating processed foods (Kuźbicka & Rachoń, 2013) results in overeating and increased energy intake. This is followed by less energy consumption due to physical inactivity and sedentary habits supported by many factors, including but not confined to urbanization, easy access and use of technology, use of automobiles, high density traffic, lack of parks, sidewalks and sports facilities (Booth *et al.*, 2017). Biologically, waning in energy consumption is primarily due to increased chemo-mechanical productivity of the skeletal muscles, whereas rise in energy intake present increased appetite and delayed satiation, and a reduced feeling of how much has was eaten (Heidlauf & Röhrle, 2014; Maher *et al.*, 2019; Polidori *et al.*, 2016). Such consequences, which do not decline over time are mostly intermediated by a continuous decrease in circulating amount of the adipocyte-derived hormone leptin, affecting either directly or indirectly through the autonomic nervous (ANS) and neuroendocrine systems (Rosenbaum & Leibel, 2014). The autonomic nervous system is further divided into sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) (Bankenahally & Krovvidi, 2016). SNS tenor manipulates eating sense through effects on various intestinal peptides and introduction of nutrient consequential signals to the brainstem (Bliss & Whiteside, 2018), and directly intensify the rate of heart beat with increased secretion of thyroid hormone (O'brien *et al.*, 2017; Joho *et al.*, 2018) whereas stimulated tone of PNS slows heart rate and decrease resting energy output (Müller *et al.*, 2016).

The human colon's resident bacteria are predominately anaerobes that salvage energy through the metabolism of ingested food ingredients and gut secretions. The major product of this metabolism are Short Chain Fatty Acids, fatty acids with fewer than six carbon

atoms (Topping, 1996). Among all types of SCFAs, the three acetates, propionate, and butyrate comprise a portion more than 90% and exist in the colon in a typical ratio of 3:1:1 (Venegas *et al.*, 2019). The production of SCFAs depends on many factors, including the type and volume of substrate, intracolonic environment, and intestinal micro-ecosystem (Macfarlane & Macfarlane, 2003; Morrison & Preston, 2016). Within the intestinal tract, some microorganisms are effective fermenter themselves, while other do fermentation either in a synergistic manner or by getting their substrates as a byproduct of fermentation, produced by other microorganisms. Also, there is a vigor microbe-microbe association tangled in the fermentation of complex nutrients. Furthermore, after SCFAs have been produced, these are exposed to high intracolonic transition (Venegas *et al.*, 2019). When produced, SCFAs are absorbed effectively by the colonocytes, leaving only 5 to 10% behind to be excreted in feces (Besten *et al.*, 2013). SCFAs have been proposed to supply nearly 10 percent of the human regular energy needs, and colonocytes mainly rely on SCFA oxidation as their primary source of energy (Baxter *et al.*, 2019; Den Besten *et al.*, 2013). Colon-originated SCFAs are then delivered to the liver as an energy source through the portal circulation. Excluding acetate that is not used by the hepatocytes, a small amount of propionate and butyrate reaches the main circulation and other tissues (Chambers *et al.*, 2018). Besides energy sources, SCFAs play an essential role in glucose and lipid metabolism directly or indirectly while acting as signaling molecules. In the colonic environment, SCFA attaches to G protein-coupled receptors GPR-41 and GPR-43 present in the endocrine L cells and stimulate the rate of intestinal hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Lu *et al.*, 2016). The GLP-1 and PYY then influence glucose homeostasis by stimulating glucose-dependent insulin release and inhibiting glucagon release from the pancreas (Steinert *et al.*, 2017). Moreover, SCFAs trigger leptin secretion in the adipocytes in response to energy depletion that in turn speeds up fat oxidation in an AMPK-dependent way both in hepatocytes and muscles (Jiao *et al.*, 2020). It also slows down the expression of glucose-6-phosphatase resulting in inhibition or reducing hepatic gluconeogenesis (Jiao *et al.*, 2020). The AMPK activates the peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α that encourages fatty acid oxidation and thermogenesis in brown adipose tissue (BAT), thus promoting calorie burning. PGC-1α also control many other transcriptional factors like peroxisome

proliferator-activated receptor (PPAR)-α, PPAR-γ, PPAR-δ, and farnesoid X receptor (FXR), all of these control glucose, lipid, and cholesterol metabolism (Cheng *et al.*, 2018; Jiao *et al.*, 2020). Due to dietary SCFAs when PPARγ get expressed it reduce white adipose tissue (WAT) mass, adipocyte size and induce peripheral insulin sensitivity (Cheng *et al.*, 2018). Further it regulates bile acid synthesis through negative feedback mechanism (Wang *et al.*, 2018). Another bile acid receptor is GPR TGR5 that induce thermogenesis and energy consumption in BAT. Both the FXR and TGR5 are presented on enteroendocrine L cells and control GLP-1 synthesis: TGR5 encourages GLP-1 synthesis, while FXR activation reduce it (Chiang *et al.*, 2017). SCFAs invigorate the production of satietogenic hormones such as leptin, GLP-1, and PYY, that are supposed to improve satiety by acting upon NPY/AgRP and POMC/CART in the ARC (Lu *et al.*, 2016). Further, the GLP-1 and PYY delay gastric evacuation and rise afferent vagal nerve triggering to the hypothalamus, assisting satiety (Lu *et al.*, 2016).

2.2.3.2 Gut microbiota, inflammation, and insulin impairment

Though it is widely accepted that mining of excessive energy from the dietary components by the intestinal microbiota results in the onset of obesity and associated disorders through various mechanism, these concepts have also explored the interaction between gut microbiota, obesity-linked metabolic diseases, and causing of low-grade inflammation. Studies suggest that inflammation may originate from macrophage intrusion into different body regions like adipose, muscle, and liver tissues, enhancing pro-inflammatory agents' excretion (Menghini *et al.*, 2007). However, actual functionality of the macrophages and the cause and kind of activating elements that provoke the immunity in this particular milieu persist a reason to comment (Menghini *et al.*, 2007). As far as concerned, the inflammatory cytokines like Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6) are often associated with the progression of diminished insulin activity and various genetic connections between insulin signaling and the immune system. For example, the c-Jun N-terminal kinase (JNK), Transcription Nuclear factor κB (NF-κB), and mitogen-activated protein kinase (MAPK) regulate pathways that have a special function in the onset of low-grade inflammation with an outcome of insulin resistance. It

is believed that fatty acids (SCFA), if consumed or produced in excess, act like pro-inflammatory cytokines themselves or induce other pro-inflammatory cytokines, leading to the activation of inflammatory cytokines and onset of inflammation (Mirmonsef *et al.*, 2012). The activation of TLRs associated innate immunity has been linked to SCFA which activates Toll-like receptor-4 (also LPS receptor) signaling in macrophages and adipocytes, leading to the inflammation of adipose tissues (Duan *et al.*, 2018). Interestingly, these molecular mechanisms act like a hub that interconnect metabolic and immune responses with each other as a result of infection by Gram-negative bacteria-derived compounds, named lipopolysaccharides (Duan *et al.*, 2018). Chronic metabolic diseases are deeply connected to low-grade inflammatory conditions and complicated interaction of receptors involved in the host-microbe communication. In this context, the Lipopolysaccharides derived from Gram-Negative bacteria have been proven a prime source of primary inflammation and metabolic diseases in human subjects, suggesting the involvement of microorganism's derived products in the onset of inflammation metabolic illnesses (Ngkelo *et al.*, 2012). Similarly, consumption of excess dietary fats increases plasma lipopolysaccharide levels and develops metabolic endotoxemia linked to the altered composition of gut microbiota (Noailles *et al.*, 2018). The type and concentration of LPS present in the body affect metabolism and immunity and determine the severity and condition of inflammation (Kell & Pretorius, 2015; Monguió-Tortajada *et al.*, 2018). Much work has been done exploring the capability of intestinal microbiota in the cause of insulin resistance and metabolic disorders associated with inflammation by modifying the plasma LPS concentrations. Based on the hypothesis that intestinal microbiota might have role in the onset of endotoxemia after consumption of fatty diet, the first study was performed on mice model. It was observed that mice fed with high-fat diet had dysbiosis in intestinal microbiota and two to three folds increased levels of plasma LPS when compared to their wild control group. Remarkably, this high-fat diet changed specific microbial species in the intestinal ecosystem, with the decrease in the number of *Bifidobacterium spp*, *Bacteroidetes, Eubacterium rectale,* and *Clostridium coccoides* (Saito *et al.*, 2007). The significance of LPS signaling in the initiation of diet-dependent inflammation was confirmed in mice-model devoted to co-receptor CD14 of the Toll-like receptor- 4 (CD14−/−mice) fed with the high-fat diet for four weeks. On completion of the trial, these

mice gained more weight and had more fats with inflammatory cytokines (signatures for low-grade inflammation) in the adipose, muscle and liver tissues. Interestingly, the mice were found resistant to diet-dependent weight gain. Inflammation and metabolic disorders in the absence of LPS receptor (Saito *et al.*, 2007). Similarly, infusion of subcutaneous LPS promoted inflammation and insulin resistance and resulted in fat deposition in subcutaneous and viscera areas with percentages of 30 to 40, respectively. The role of microbial LPS in exploding inflammation, insulin resistance, type 2 diabetes and metabolic disorders have been tested in both dietary made and genomically persuaded obese mice by modulating certain microorganism of the intestinal ecosystem (Do *et al.*, 2018; Membrez *et al.*, 2008; Zhang *et al.*, 2018). When broad spectrum antibiotics were given to the diet induced obese mice in order to stop the growth of intestinal microbiota, this prevented the mice from getting fat, development of inflammation, insulin resistance and oxidative stress, proposing that high fat might not be a direct cause for weight gain and onset of obesity. While genetically obese mice showed improvement in insulin resistance and decrease in inflammation without reducing body weight (Cani & Delzenne, 2011). To sort out the exact role of LPS in the onset of obesity via low-grade chronic inflammation and insulin resistance, Canis *et al* interfered LPS signaling in two mice models. In one model polymyxin B (LPS quencher) were infused subcutaneously for a duration of four moths in intrinsically persuaded obese ob/ob mice while the other obese mice were missing the CD14, LPS receptor (ob/ob CD14-/-). Significant decrease in inflammatory cytokines, macrophages infiltration, improved glucose tolerance and insulin resistance were observed in both models (Cani & Delzenne, 2011). Moreover, polymyxin B exposure, which precisely eradicates Gram's negative bacteria, suppress LPS level and reduce hepatic steatosis (Pappo *et al.*, 1991). These results strongly support the concept that intestinal microbiota pay to the metabolic endotoxemia associated to both inherent and diet-produced obesity.

The link between high-fat diet, LPS, low-grade chronic inflammation, obesity, and type 2 diabetes has been investigated and confirmed in healthy and obese humans. It has been found that increased or frequent consumption of a high-fat diet and energy-dense food induces 2-3 folds more metabolic endotoxemia and inflammation in healthy subjects (Amar *et al.*, 2008; Mo *et al.*, 2020; Quintanilha *et al.*, 2020). Metabolic endotoxemia raises

inflammatory cytokines, specifically adipose TNF-α and IL-6 resulting in insulin resistance, type 2 diabetes, obesity, and metabolic syndrome in healthy subjects (Awoyemi *et al.*, 2018; Boutagy *et al.*, 2016). Similarly, a high blood circulating LPS caused adipose tissue impairment in obese human subjects (Clemente-Postigo *et al.*, 2019). The endotoxic state of obese patients is directly inversely correlated with plasma triglycerides and cholesterol levels while inversely correlated with glucose, and insulin (Al-Attas *et al.*, 2009; Guevara-Cruz *et al.*, 2019; Radilla-Vázquez *et al.*, 2016). Recent information supports the idea that activation of the LPS-TLR-4/CD14 complex is a pre-requisite of inflammation development. This also suggests that TLRs have a prominent role in developing low-grade inflammation, insulin sensitivity, and obesity. Finally, different studies have ruled out TLR-2 and TLR-4 in the development of chronic inflammation, obesity, and metabolic diseases (Kuo & Yu, 2011; Rogero & Calder, 2018). The TLR-2 recognizes many lipid-containing molecules with bacterial lipopeptide, and therefore, the initiation and expression of TLR-2 have a direct dependency on LPS expression, but also on TNF-α and CD14 (Dumitru *et al.*, 2000; Hanzelmann *et al.*, 2016). This finding describes that up-regulation of TLR-2 in the existence of microbial's LPS is a central procedure through which the host's defensive response increased highly to microbial infection (Babcock *et al.*, 2006). It can be said that metabolic endotoxemia enhances the expression of TLR-2, increasing the LPS/TLR-4/CD14 signaling to provoke inflammation. Some other studies suggested that lipids/fatty acids develop minor inflammation vis TLR-4 depending on way (Lancaster *et al.*, 2018; Suganami *et al.*, 2007). It can be concluded that fatty acids are indeed responsible for activation of the immunity system but may be jointly with early activation by LPS of the TLR-4/CD14 resulting TLR-2 activation. Different early studies have confirmed the role of TLR-4 and TLR-4 in the development of obesity. Like modulating the intestinal microbiota with the use of antibiotics protected mice from dietary produced obesity, equally in the existence of active TLR-4/2 receptors (Cani *et al.*, 2008). In the same way CD14 (required for proper activity of TLR-2 and TLR-4) knock-out mice did not develop fat-induced low-grade inflammation, even TLR-4 and TLR-2 receptors were completely active (Roncon-Albuquerque *et al.*, 2008). When fed a high fat diet and allowed to fully absorb and digest fats, the germ-free mice did not show any signs of inflammation (Rabot *et al.*, 2010). Tying together, this research proposed that

a signaling chain initiates via LPS/TLR-4/CD14-relaint way that sequentially stimulates TLR-2 to enhance native immune system inflammatory action.

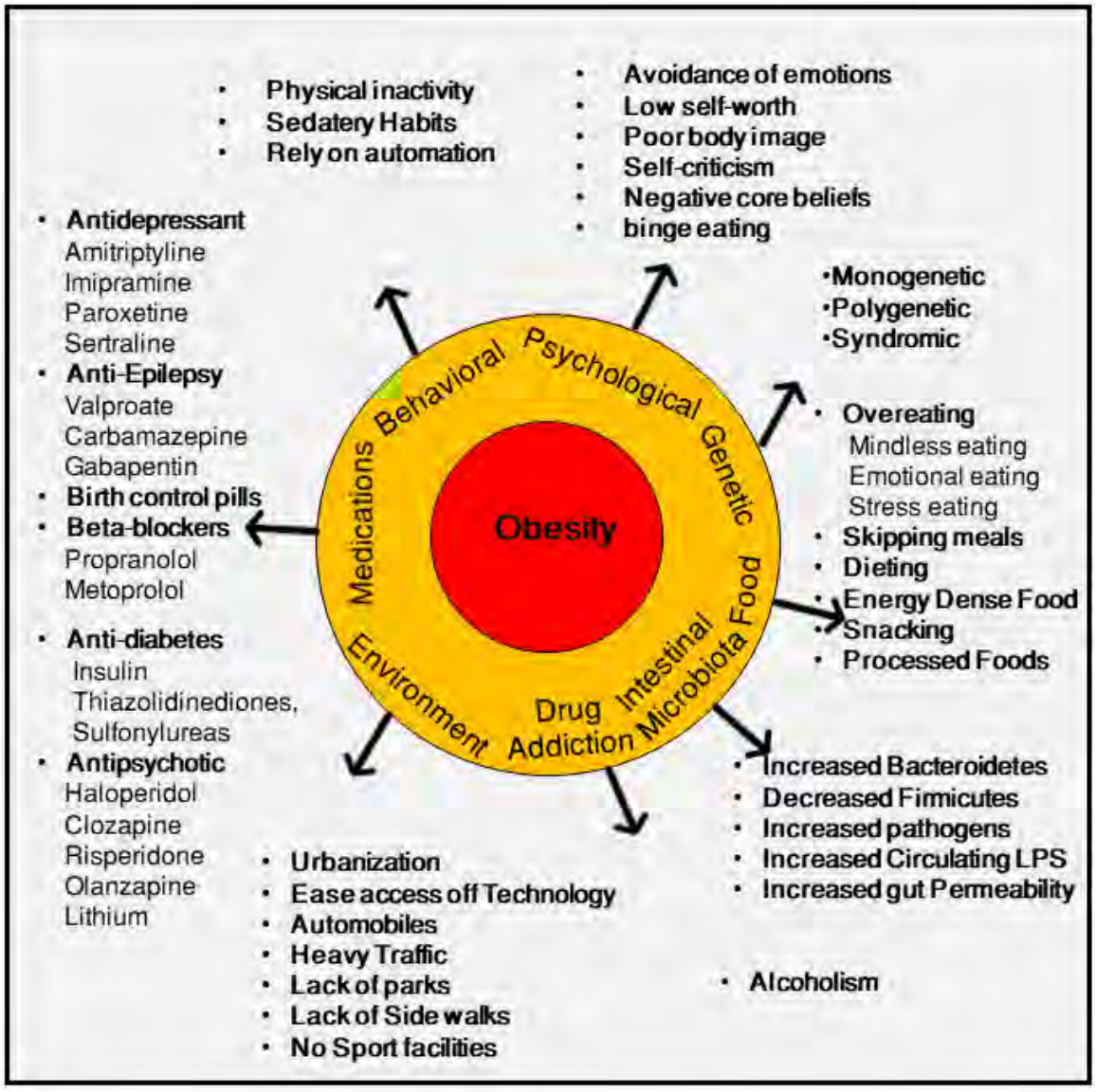

Figure 2. 5: Factors involved in Gut microbiota Dysbiosis.

2.2.3 Gut microbiota and leaky gut

Studies have been conducted to explore the mutual interaction between host and bacteria that maintain and regulate gut barrier functions (Hayes *et al.*, 2018). Many studies have linked dysbiosis in intestinal microbiota with the onset of metabolic endotoxemia and inflammation (Macfarlane & Macfarlane, 2003). In normal healthy physiology and balanced gut microbiota, the intestinal epithelium works like a most substantial, and regular

barrier preventing bacterial translocation. The epithelium barrier comprises three complex junctions: tight junction, adherens junction, and desmosomes. The tight junction is the apical most adhesive complex that mainly seal the intercellular space (Vancamelbeke *et al.*, 2017). The tight junction is consists of transmembrane proteins (like claudins, occludin), peripheral membrane proteins (like zonula occcluden ZO-1, ZO-2), and regulatory proteins (Vancamelbeke *et al.*, 2017). Numerous endogenic and/or exogenic conditions are involved in the disruption of this defensive function. Some well-known contributors which result in the leaky gut are immobilization stress (Jang *et al.*, 2018), alcohol consumption (Meroni *et al.*, 2019), radiation, and use of some drugs and medicines (Garg *et al.*, 2016). The two tight junction proteins Zonula Occuldens -1 (ZO -1) and Occludin, when altered in structural configuration or positioning, are known to be linked with increased gut permeability and are documented in diabetes and obese animal models (Lee *et al.*, 2018). Different mechanisms have developed to connect the obesity gut microbiota dysbiosis with disruption in the intestinal barrier. While modulating the intestinal microbiota of native obese mice resulted in significant healing of gut penetrability when assessed *in-vivo*; that is associated with improved tight junction mRNA transcription and amended protein organization. Which further led to reduced circulating plasma LPS concentrations and inflammatory cytokines (Cani *et al.*, 2009). It has been evaluated that feeding mice with prebiotics results in increased synthesis of glucagonlike peptides (GLPs) by the L-cells mainly GLP-1 and GLP-2. The GLP-1 production is linked with differentiation and maturation of endocrine cells while GLP-2 is associated with synthesis and distribution of junction's proteins. Synthesis and expression of these peptides improve the integrity of gut barrier and hence reduce endotoxemia (Dubé *et al.*, 2007) (Figure 2.6).

The Endocannabinoid system (eCB) also has a meaningful effect on the gastrointestinal microbiota in the development of obesity where the level of CB1 and eCB changes in the body. The eCB system is especially expressed in the intestinal and adipose tissues (Lee *et al.*, 2018). The CB1 receptor has been linked to the control and regulation of intestinal integrity. For example, hindering the CB1 receptor in mice decreases intestinal absorbency through enhanced tight-junction proteins (ZO-1 and Occludin) dispersal and localization. Adding, CB1 stimulation raises permeability indicators when measured in *in-vivo* and *in*

vitro (Lee *et al.*, 2018; Mehrpouya-Bahrami *et al.*, 2017). These experiments proved that CB1 receptors have a role in regulating gut permeability via communications with the intestinal microflora (Mehrpouya-Bahrami *et al.*, 2017). It has been established that crosstalk between eCB and gut microbiota regulate the rate and mechanism of adipogenesis (Cani & Infection, 2012; Lee *et al.*, 2018). Significantly, it has been found that modulating the intestinal microbial ecosystem through prebiotics stabilizes eCB system both in the intestine and adipose tissues, and these changes are actively connected to the reduction in intestinal permeability, metabolic endotoxemia, and fats growth. However, it would be distinguished if solid links persist among the structure of gut microbiota and factors regulatory to gut barrier activities, the direct contribution of certain intestinal microbes and/or microbial products remain to be clarified. The structure of tight junction is presented in Figure 2.6.

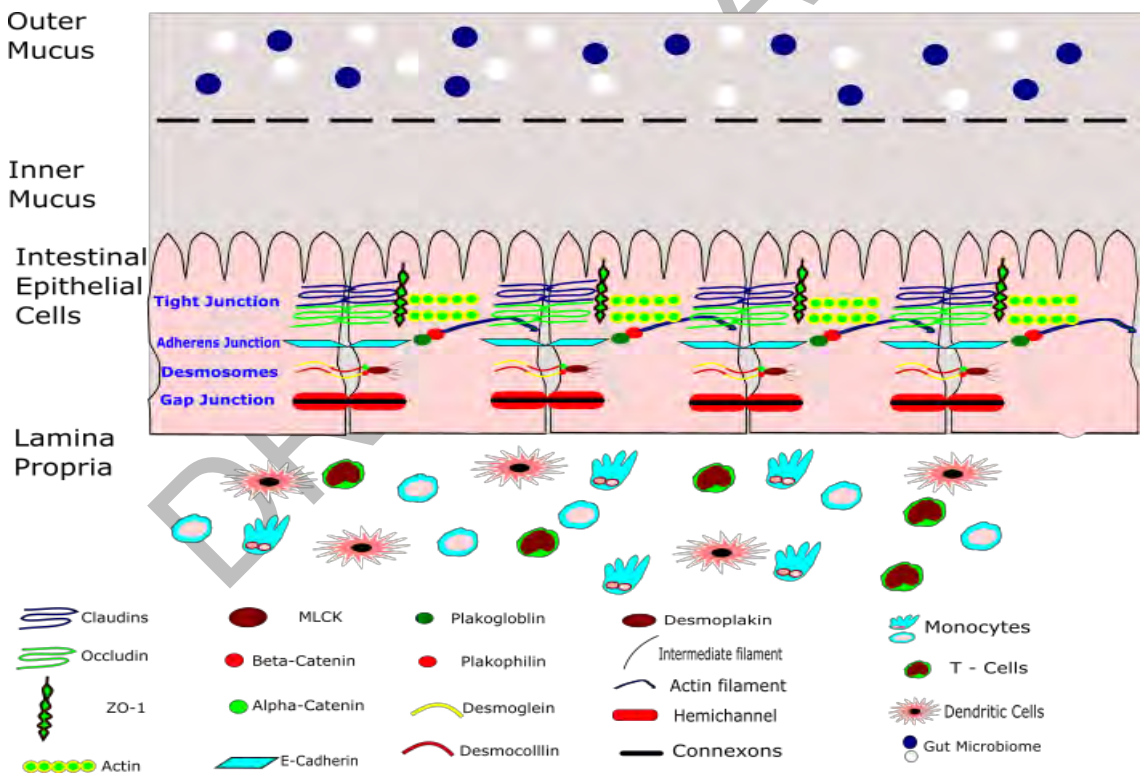

Figure 2. 6: Structure of Intestinal Tight Junction. The schematic diagram illustrates structure of an intact intestinal junction, it also highlights it key functionalities in terms of immunity and homeostasis.

2.3 Lactic acid bacteria as probiotics

2.3.1 Introduction

Thousands of years ago, Hippocrates a Greek philosopher known as the father of medicine, said about food, "*Let food be thy medicine, and let medicine be thy food."* And then, Chow, in 2002, accepted this view based on experimental work and suggested that food could be used as medicine. However, at present, foods with therapeutic effects have been termed as 'Functional Foods.' The number of healthy characteristics associated with functional foods are increasing day by day, and the gastrointestinal tract is the main target for testing and development of functional foods because it is a middle ground between diet and all the functions of the body. The best area for producing functional foods is pre and probiotics, which have scientific evidence of health-linked properties. Today, the public is well aware of the relationship between food, lifestyle, and health, which enlightens the increasing need for food items that could improve health status beyond having elementary nutrition. Ingestion of fermented foods has been suggested to provide numerous health, such as improving the immune system, resistance to malignancy, prevention of infectious diseases, and essential nutrition. These properties of fermented foods have been linked to microorganisms, especially a dominant group, lactic acid bacteria, present in them (Szutowska & Technology, 2020). Lactic acid bacteria were first isolated from milk and have been present in many other fermented products such as milk, beverages, bakery, and meat. LAB is ubiquitous and is present in many environments like water, sewage, soil, manure, plants, and silage. They are the normal flora of humans and animals residing on the mucous membranes of the urinogenital tract, gastrointestinal tract, mouth, and skin and positively affect these aforementioned ecosystems. Lactic acid bacteria that grow in the food as their indigenous flora or those added as adventitious cultures have no harmful effect but even, they are advantageous for the consumers (Szutowska & Technology, 2020).

Meanwhile, their detection, LAB has attracted much attention for its use in numerous applications such as starters for fermentation, biotherapeutics, probiotics, and bio-preservation. LABs are widely used in the food industry as starter cultures to achieve desirable texture, taste, flavor, and aroma (Landete, 2017). At the same time, non-fermentative LAB with antibacterial peptides producing ability is used as bio preservatives. Due to their antimicrobial and antioxidant activities, some LAB strains are used in food

bio-preservation (Landete, 2017). Though LAB is generally regarded as safe (GRAS) for the user, and when stored, they generally exceed microorganisms of various food products (Plavec & Berlec, 2020). Several properties associated with probiotic functionality have been originated from the effects examined in clinical trials. Even a limited number of strains have formally earned a place of biotherapeutic composition; each is regularly tested through numerous experimental tails or clinical interventions, conducted in such a way that bear a resemblance to the conventional pharmaceutic approaches (randomized experiments, placebo trails, double blinds) and the strains applied in such trails are from different species. However, the majority are lactic acid bacteria (Plavec & Berlec, 2020).

2.3.2 LAB as probiotic candidates

2.3.2.1 A short sketch of probiotics

The utmost widely used and recognized method through which intestinal microorganisms can be affected is using bacterial dietary inclusions as probiotics. The idea is centuries old, and the first-time humans ingested viable bacteria was more than 2000 years old. However, for the first time since Metchnikoff's work in 1908, scientific experiments on probiotics began. He proposed that microorganisms typically present in the intestine harmed human health, which was reversed by consuming soured milk. The term "Probiotic" was first practiced as a pseudonym for the term "Antibiotic." This Greek word means "for life." The first use of this word can be associated with Kollath (1953), who used it to define the restoration of the well-being of undernourished subjects with various kind of natural supplements. Later in 1954 Vergin suggested that probiotic diets could have reversed intestinal microbial dysbiosis caused by use of antibiotics; an idea quoted by many as the first-ever reference to probiotics as they are described today. In the same way, Kold identified the adverse effects of antibiotics and suggested prevention using probiotics. Later, Lilly et al., (1965) described probiotics as "microorganisms promoting the growth of other microorganisms" resulted in the approval of a FAO/WHO (2002) group on the assessment of probiotics in food as probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host (Schrezenmeir & Vrese, 2001; Vasiljevic & Shah, 2008).

The concept that LAB has positive effects on human health is old; as Metchnikoff suggested that the lactobacillus group might prevent gut microbial dysbiosis and prolong life. These bacteria may not essentially be indigenous to the intestine but must confer a "beneficial effect on the general and health status of man and animal" (Chen *et al.*, 2020). Additional descriptions through time have been confined through the designation of action, position of entry, carriage form/way, or the host. Probiotics have a wide range of positive effects on the host. In connection to food and diet, probiotics are the "viable preparations in foods or dietary supplements to improve the health of humans and animals". According to this definition, a striking figure of bacteria are counted as probiotics (Sanders *et al.*, 2018). However, the most vital bacteria that are used as probiotics for the gut environment belongs to lactic acid bacteria (LAB) (Harzallah & Belhadj, 2013)

2.3.2.2 Selection of probiotics

Several *in vitro* assessments are conducted while selecting potent probiotic isolates. The primary stage in screening probiotic LAB isolates is the taxonomic catalog decision, which may be used as a gesture for source, habitation, and physiochemistry. These properties are significantly influenced in the selection of new isolates (Shokryazdan *et al.*, 2017). The FAO/WHO (2002) professional board advised that the particularity of probiotic action is more significant than the origin of the isolate. This decision was made because of the ambiguity in the source of intestinal microbiota meanwhile, the newborns are born with almost no intestinal flora. Nevertheless, the panel also clarified the necessity for the perfection of *in vitro* tests to guess the activity of probiotics in humans (Shokryazdan *et al.*, 2017). Although several probiotics justify the standards like tolerance to acids and bile salts, persistence through intestinal transit, a model probiotic candidate still needs to be tested for other needed characteristics. Moreover, it is questionable that a single strain will be appropriate to all the required parameters; opting strains for disease-specific assessment will be needed (Pereira *et al.*, 2018).

The primary characterization for probiotic selection includes assessment of the following vital criteria: phenotypic and genotypic (including plasmid) stability; degree and pattern of amylolytic and proteolytic activities; acid and bile resistance and survival and growth; intestinal epithelial adhesion properties; release of antimicrobial compounds; susceptibility

to antibiotics; antipathogenic ability; and immunogenic ability. The ability of probiotics to stick to mucus membrane is one of the main selection criteria as adherence to the intestinal lining is the prerequisite for colonization (De Melo Pereira *et al.*, 2018). Figure 2.7 displays key while basic steps for selecting probiotic candidates.

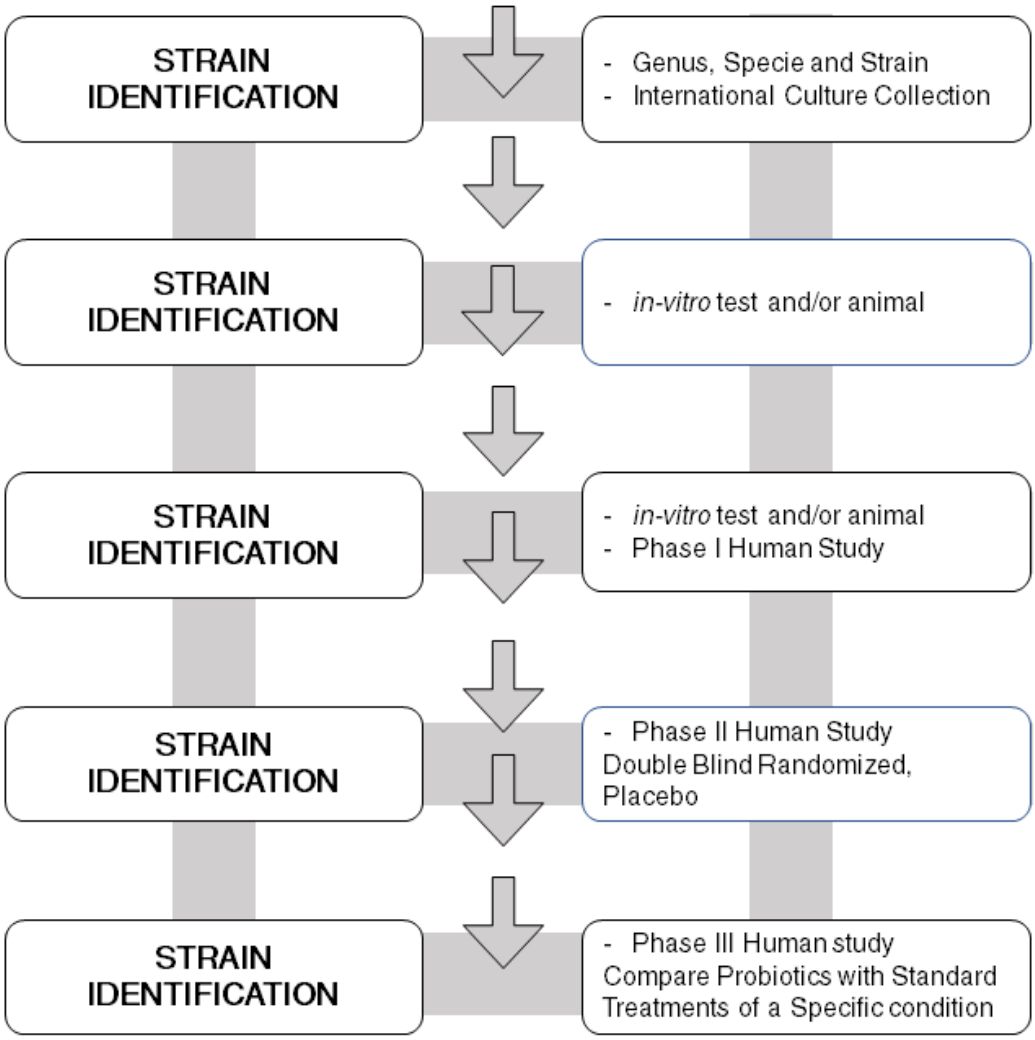

Figure 2. 7: Key steps involved in selecting probiotics for commercial use (Harzallah & Belhadj, 2013).

It is imperative that probiotic candidates live where it is supposed to be active. For an extended period and efficient performance, the isolate must colonize and multiply at a specified site. Hence, it can be concluded that host-specific strains would withstand the competitive pressure exerted by indigenous flora and colonize the site for growth and multiplication (Zuo *et al.*, 2019). Also, the probiotic isolate must not be taken as an

unwanted foreign body by the host's immunity and must not trigger the development of any antibodies. Moreover, a probiotic isolate can act as immunogenic and activate immunity against infectious microorganisms. It can be concluded that an administered probiotic isolate has not to have adverse effects on its host: no localized or systemic infections, averse or carcinogenic/mutagenic effects initiated by probiotic, its metabolic byproducts or cellular elements that may produce as a result of probiotic demise (Kothandaraman *et al.*, 2019). When probiotic isolate is chosen, the prime potency characteristics and technological basis must be evaluated. Basic early depiction of identification and taxonomic classification of the strain should be confirmed, subsequently by assessment with confirmed analysis both in animal models and in controlled pre-clinical and clinical trials (Sanders *et al.*, 2018). Primarily the *In vitro* analyses are performed that are not prognostic to the *in-vivo* assessments. The strain must be evaluated for some significant technological properties like its potentials to proliferate efficiently, assiduous, stabilized, and integrated in the product with the best desirable characteristics. The strain must be tested for its physical and genetical stability over the product's shelf life and at a specified location in the host. Analysis of the strain's stability can be tough since aspects such as colony size and rupture may affect the typical evaluation of CFUs and *in-vivo* functions (Schrezenmeir & Vrese, 2001). The dosage of probiotic isolate must be based on the quantity tested to be efficient in clinical trials as one dosage cannot be functional for most isolates. However, the effect of composition and structure of the final product on the efficacy of probiotics has yet to be investigated. General quality-control standards of cfu/g may not be the actual limit talking about the efficiency of the final product. Many other features, like strain's proliferation during production, packaging, and preservation of the product, metabolism of the strain, and the existence of many other effecting constituents in the product, may define efficacy of the product (Sanders *et al.*, 2018).

2.3.3 Possible means of action of probiotics

A broad range of health benefits has been linked with the use of probiotics**.** The declared benefits range from mitigation of dyschezia to preclusion of lethal illnesses like obesity, inflammatory bowel disease (IBD), diabetes, cancers, and cardiovascular diseases. Few proposed benefits, for example, the decrease of intestinal transit or comfort for lactose

intolerance, are authenticated, while others, such as anti-cancerous, anti-diabetic, or their role in beneficial modulation of the gut microbial ecosystem, need further experimental work (Szutowska & Technology, 2020). The mechanisms of action strain-specific and, mostly, perhaps a combination of activities, hence exploration of the desired action is a challenging, and intricate job. Generally, three types of action can be exerted by probiotics: influence hot's health by interacting with the host's indigenous microflora, firming mucosal linings, and modulating the immunity (Leroy *et al.*, 2008). Figure 2.8 present main mechanisms through which the probiotic exert its effect in the gastrointestinal tract.

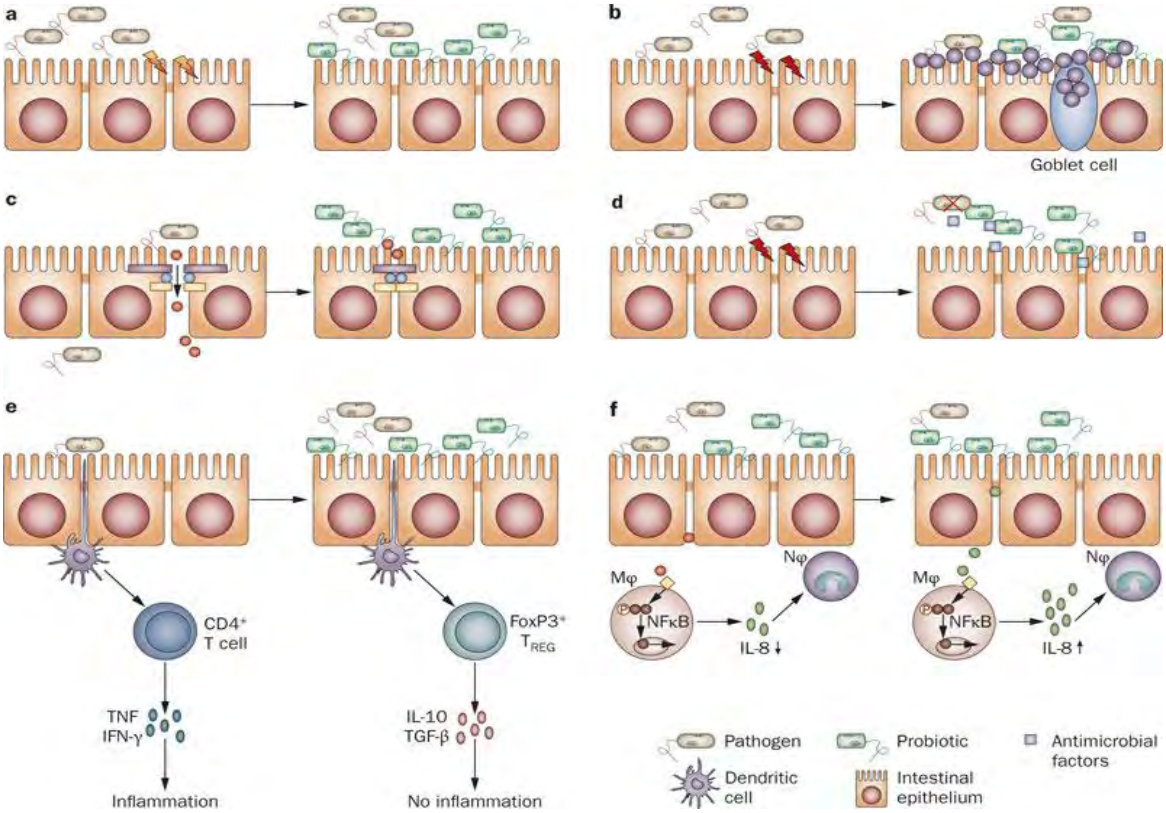

Figure 2. 8: Potential mechanism of probiotics in the gut adopted from (Harzallah & Belhadj, 2013).

2.3.4 Probiotics and intestinal health

2.3.4.1 Gut microbiota

The human intestinal tract is a habitat to more than 1000 different types of bacterial species living in an outstanding balance. These species mainly belong to the *Bacteroides*, *Lactobacillus*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia, and Veillonella* genus. It has been proposed that this

complex ecosystem occupies 30-50% volume of the human colon (Park *et al.*, 2017). The established health benefits of the probiotic bacteria so far are *Bifidobacterium* and *Lactobacillus* species (Redondo-Useros *et al.*, 2019). It has been observed that the dominant intestinal bacterial communities remain constant during adultness, and the bacteriological pattern for everyone is as unique as fingerprint itself. But there are many factors that can affect the composition of microbial communities in the gut, including genetics of the host, delivery mode, dietary pattern, age, exposure to antibiotics, and other microbes as probiotics (Crovesy *et al.*, 2020). The intestinal tract is among the main areas of interaction with external objects, including allergens, viruses, bacteria, and other infectious entities in the human body. The intestinal tract acts as the primary means of host defense against exogenous attacking objects through intestinal mucosa, localized immunes system, and synergistic interaction with gut microbiota. Intestinal microbiota promotes human health through gut epithelium barrier, immune system modulation, and nutrient metabolism (Crovesy *et al.*, 2020). A barely small fraction of bacterial phyla constitutes a significant portion of intestinal microbiota, and it has been reported that 80% of microbiota belong to *Clostiridium leptum*, *Clostridium coccoides*, *Bacteroides,* and *Bifidobacteria* in the adult human body.

Moreover, this dominant fraction is also unique per study subject. Research has shown that, though subject specific, but mucosal microflora is constant through the distal intestinal tract from the ileum to rectum, but 50 – 90 % different from fecal flora. The number of intestinal microorganisms is not the same throughout the entire gastrointestinal tract. It has been observed in continuum as the stomach and duodenum carry 10^1-10^3 bacterial cells/gram of contents, followed by 10^4-10^7 bacterial cells/g in jejunum and ileum, concluding in 10^{11}-10^{12} cells/g in the colon Figure 2.9(a). Along with lengthwise variation in intestinal microflora, there is also heterogenicity in widthwise microbial constitution Figure 2.9(b). The epithelium is supported from lumen with dense, complex gunk lining and hence the lumen microflora drastically varies from those attached or rooted in mucus layer and from the epithelium proximal microflora. For example, the species of *Bacteroides***,** *Bifidobacterium***,** *Enterobacteriaceae, Enterococcus, Lactobacillus, Rumincoccus,* and *Streptococcus* has been detected in fecal material, while only *Clostridium, Enterococcus,* and *Lactobacillus* has been present in the crypts of epithelium

and mucus layer of the small intestine (Sekirov *et al.*, 2010). It has been proposed that the human gut starts inoculated with microorganisms soon after birth (Figure 2.9 (c). During delivery, the infant is exposed to complex and microbiota as it passes through the birth canal. From birth to the first year of life, human gut microbiota is quite simple and usually differs between various subjects and time. After one year, the infant's gut microbiota begins to stabilize and resembles the adult's gut microbiota Figure 2.9 (c) (Sekirov *et al.*, 2010).

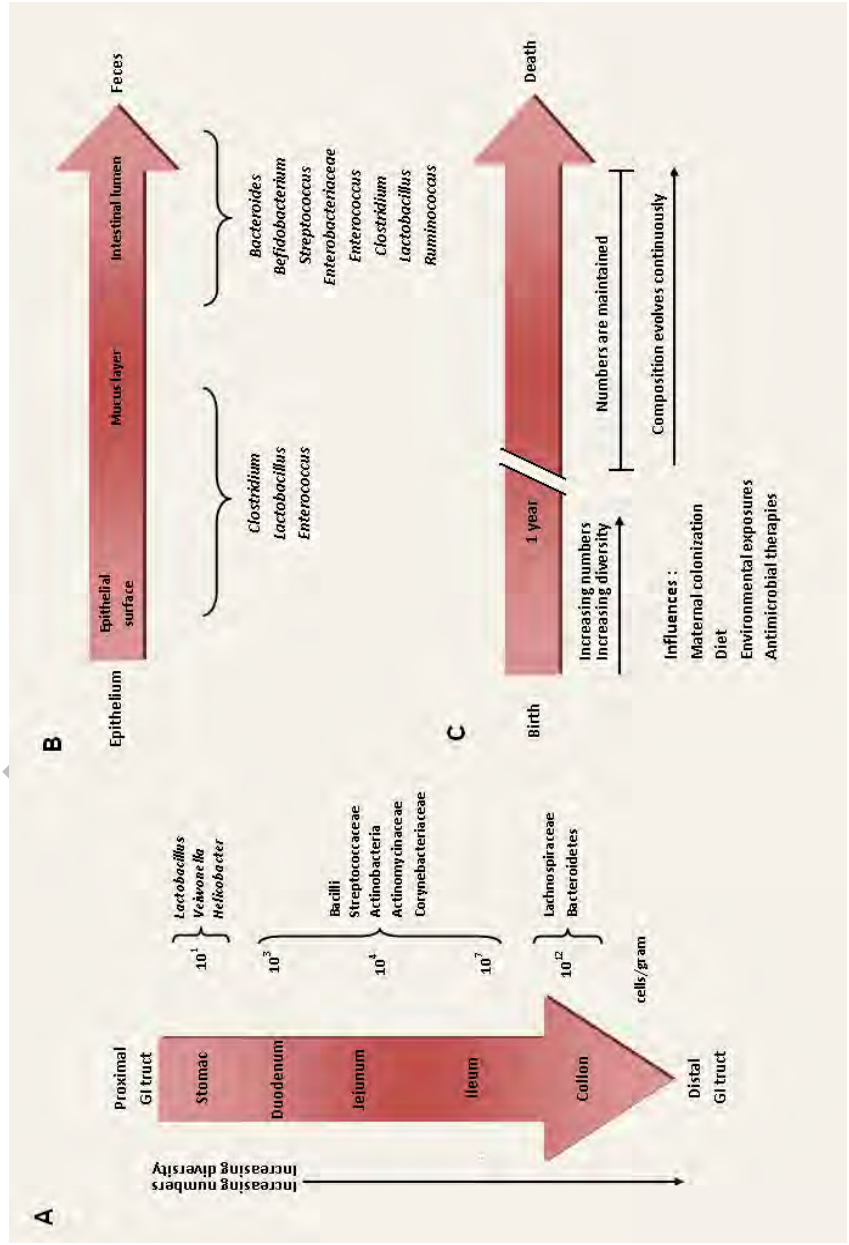

Figure 2. 9: Composition of gut microbiota adopted from (Harzallah & Belhadj, 2013).

2.3.4.2 Survival and antagonistic effect of probiotic in GIT

The epithelium works like a rigid blockade to foreign microbial aggressors and is also implanted with numerous defensive objects of the naïve immunity. The intestine's two key elements have a vital role in interacting host and external triggers: intestinal permeability and gut mucosal immunity (Fata *et al.*, 2018). Resident microbes of the gut can inter-react with invading pathogens and antigens to secure the gut using several mechanisms. Based on a general explanation of probiotics, the probiotic microbe should be alive when consumed to deliver health benefits. So, it would not be wrong to say that a probiotic should sustain during passage through the gastrointestinal tract and inhabit the epithelium lining and around. Numerous mechanisms are supposed to be associated with survival in the gastrointestinal tract during the passage, the most vital and accepted of which is resistance to very low gastric pH and various bile salts of the intestinal tract. Such characteristics are therefore known significant selection measures for novel probiotic use.

Along with tolerance to asperous conditions of the GI tract, attachment to intestinal mucosa would be needed for colonization and synergistic interaction of probiotics and host resulting in competing for the elimination of pathogens and/or mitigation of host cell response (Fata *et al.*, 2018). A well-known example of this condition is enteropathogenic *E. coli* that attaches to the epithelium through mannose receptors; probiotics with the same attachment properties could prevent pathogen binding and growth in these locations and hence protect the host from infection (Katsowich *et al.*, 2017). Probiotics can inhibit pathogens by lowering pH, preventing their attachment and translocation, or releasing antipathogenic substances and defensins. One way intestinal microbiota prevents pathogens colonization is by creating a harsh living condition, with reduced pH, increased redox potential, and production of hydrogen sulfide (Sanders *et al.*, 2018; Sekirov *et al.*, 2010). Probiotics reduce pH in the lumen, as has been tested in subjects suffering from ulcerative colitis (UC), resulting consumption of probiotic dose VSL#3 (Chun-Sai-Er *et al.*, 2018). In another example, in a mouse model, infected with deadly, Shiga toxin-producing *E. coli* O157:H7 *Bifidobacterium breve* formed a high level of acetic acid, subsequently reducing the lumen pH, resulting in a high level augmented survival of the infected mice (Aw & Fukuda, 2019). Synthesis of antimicrobial substances, generally

known as bacteriocin, by probiotics also seems to have a role in their health-related characteristics.

Many types of bacteriocin released by *Lactobacillus* species have been identified and characterized. The antagonistic functionality of these antibacterial peptides differs; some of these bacteriocin kill taxonomically interrelated Gram-positive bacteria, and some antagonize a wide variety of Gram-positive bacteria, Gram -negative bacteria, yeasts, and molds (Field *et al.*, 2018). Such as an antimicrobial peptide produced by *L. salivarius subsp. Salivarius* UCC118 kill a variety of pathogenic bacteria, including *Bacillus*, *Enterococcus, Listeria, Salmonella*, and *Staphylococcus* (Field *et al.*, 2018). Another bacteriocin, Lacticin 3147, produced by *Lactococcus lactis* is known to kill a series of genomically diverse *Clostridium difficile* species isolated from healthy and IBD patients. Bacteriocin produced by *Lactobacillus* species inhibits *Helicobacter pylori* that cause infection of gastrointestinal mucosa (Field *et al.*, 2018). Probiotics can decrease the epithelium damage that is exposed to *E. coli* O27:H6 and *E. coli* O157:H7. The pathogenic *E. coli* could not insert its virulent factors or damage the tight junctions of T84 intestinal cell lines when these were treated with lactic acid bacteria before infecting with pathogens. Similarly, attachment and damage of Intestine 407 cell lines with adherent invasive *E. coli*, originally from Crohn's disease, was significantly reduced when co/pre-incubated with probiotic isolate *E. coli* Nissle 1917 (Field *et al.*, 2018). From these results, it can be concluded that probiotics hinder epithelial damage caused by adhesive pathogens, pay to an enhanced mucosal wall, and offer sources of preventive contact of enteric pathogens (Field *et al.*, 2018; Fata *et al.*, 2018).

2.3.5 Probiotics and the mucous membrane

Most of the bodily mucosal exteriors are shielded by a viscous type of gel produced mainly by mucins. The Mucins are formed and released particularly by epithelial cells, for example, gastric foveolar mucous cells and intestinal goblet cells, Goblet cells are present laterally on the whole length of the gastrointestinal tract and other mucosal exteriors. The glycosylated Mucins are either embedded into the membranes or released into the lumen to form a layer (Ma *et al.*, 2018). Among the total 18 glycosylated human mucins, the glycoprotein MUC2 is dominantly found in the mucus layers of small and large intestinal

tracts. Its NH2- and COOH-termini are not somehow glycosylated but are rich in cysteines that create intra- and inter-molecular disulfide bonds. These glycan clusters are resistant to any proteolytic activity and make the mucin hydrophilic, while the disulfide lineages create a mesh network of glycoprotein that is the backbone of the mucous layer (Schneider *et al.*, 2018). The glycosylated mucus layer activity allows small molecules to pass through but restricts the passage of large molecules that form a frigid fluid layer at the surface of the epithelium. As the frigid layer is prevented from convective mixing, ions' dispersion and small solutes decreases (Ma *et al.*, 2018). The mucus layer protects the intestinal tract from harmful antigens, molecules, and microbes by providing a shield. Furthermore, it acts as a lubricant for intestinal mobility. Besides this, Mucins binds to the carbohydrates present on the surface of epithelial cells, resulting in the formation of a bottom layer in such a way that it is tightly connected to the mucosa, while the upper layer is loosely attached. The mucus layer is the first-ever barrier that the intestinal microbiota has to encounter, and pathogens must breach it to access the epithelial cells during infection (Schneider *et al.*, 2018).

Probiotics are believed to increase the rate of mucus secretions as a way for the improvement of barrier activity and exclusion of infectious entities. This concept has been evidenced in *in vitro* experiments. Many studies reported high mucin production in human intestinal cell lines like Caco-2 and HT29, preventing the adherence and invasion of pathogenic *E. coli*. But this effect is directly related to the adhesion of probiotics to intestinal cell lines' monolayers, which is probably not possible *in vivo* experiments (Fata *et al.*, 2018). While, on the other hand, a study confirmed that cell extract of *L. acidophilus* A4, independent of attachment, increased MUC2 expression in HT29 cell lines (Kim *et al.*, 2008). Moreover, intestinal trefoil factor 3 (TFF3) is co-expressed with MUC2 by colonic goblet cells and is proposed to repair intestinal damage (Ma *et al.*, 2018). However, when VSL#3 probiotics were given to healthy rates, it did not increase colonic TFF3 expression (Chun-Sai-Er *et al.*, 2018). Additionally, dextran sodium sulfate-induced colitis mice did not show increased TFF3 production nor mend the damages when treated with VSL#3 probiotics. These experiments show that up-regulation of TFF3 is not associated with probiotic's activity. Hence use of these probiotics is helpful for anti-inflammatory activities in animal models (Schneider *et al.*, 2018).

2.3.6 Interaction of probiotic bacteria with the gut epithelium

, The structural configuration of normal intestinal microbiota is possibly affected jointly by the diet and other features like geography, hygiene, and climate. The interaction of microorganisms with their host is significantly crucial for newborn and then during neonatal age. The development of normal microbiota helps in immune system development and maturation by challenging through the antigens. The anti-inflammatory characteristic of intestinal immunity is mainly developed by normal intestinal microbiota. Numerous immune controlling features like apoptosis, cytokines, and regulatory cells regulate immune reflexes by inhibiting the pathological procedures linked with hyperreactivity. A significant property of probiotic activity is its ability to stimulate and regulate the immune system. Research has been done to evaluate the role of probiotics in the modulation and maturation of the immune system. Like, after taking probiotics, their interaction with gut enterocytes provokes a host response. Hence enterocytes release numerous immune regulatory molecules when interacted with bacteria (Carabotti *et al.*, 2015). Moreover, normal intestinal microflora is a native resistant to many possible pathogens and promotes colonization resistance, called intestinal barrier, via inhibiting attachment and growth of pathogenic microorganisms. Normal microbiota is believed to interfere with pathogen's adhesion and toxin and protect their host (Yan & Polk, 2020). The tight junction of the intestinal epithelium forms an additional protective line between the host and the gut luminal contents. This lining is supposed to be capable of responding to infections through introducing any non-specific or specific immune response (Krishna *et al.*, 2013). Pathogen attachment to the host cell or recognition by the host cell is mainly a necessary initial step in the infection mechanism. Like numerous glycoconjugates, many intestinal cell surface elements can act as receptors for bacterial attachment (Yan & Polk, 2020). Epithelial cells also present Toll-like receptors (TLR) as host pattern recognition receptors (PRRS): a family of transmembrane receptors responsible for recognizing repetitive patterns such as the pathogen-associated molecular pattern present in both Gram-negative and Gram-positive bacteria. There are mainly two types of TLRs, TLR2 which recognizes several microbial elements like peptidoglycan and lipoteichoic acid, from Gram positive bacteria, and TLR4, which recognizes lipopolysaccharides and Gram-negative bacteria. Besides these, other TLRs with defined functions are also known; for example, TLR5 recognizes

explicitly bacterial flagella, TLR9 responds to short DNA components having CpG sequences (Carabotti *et al.*, 2015). The receptor nucleotide-binding oligomerization domain proteins are present in the host's cell cytoplasm, recognize both Gram-positive and Gram-negative bacteria, and induce defensin production. Compromised intestinal barrier permeability is mainly linked with disease inception and perseverance.

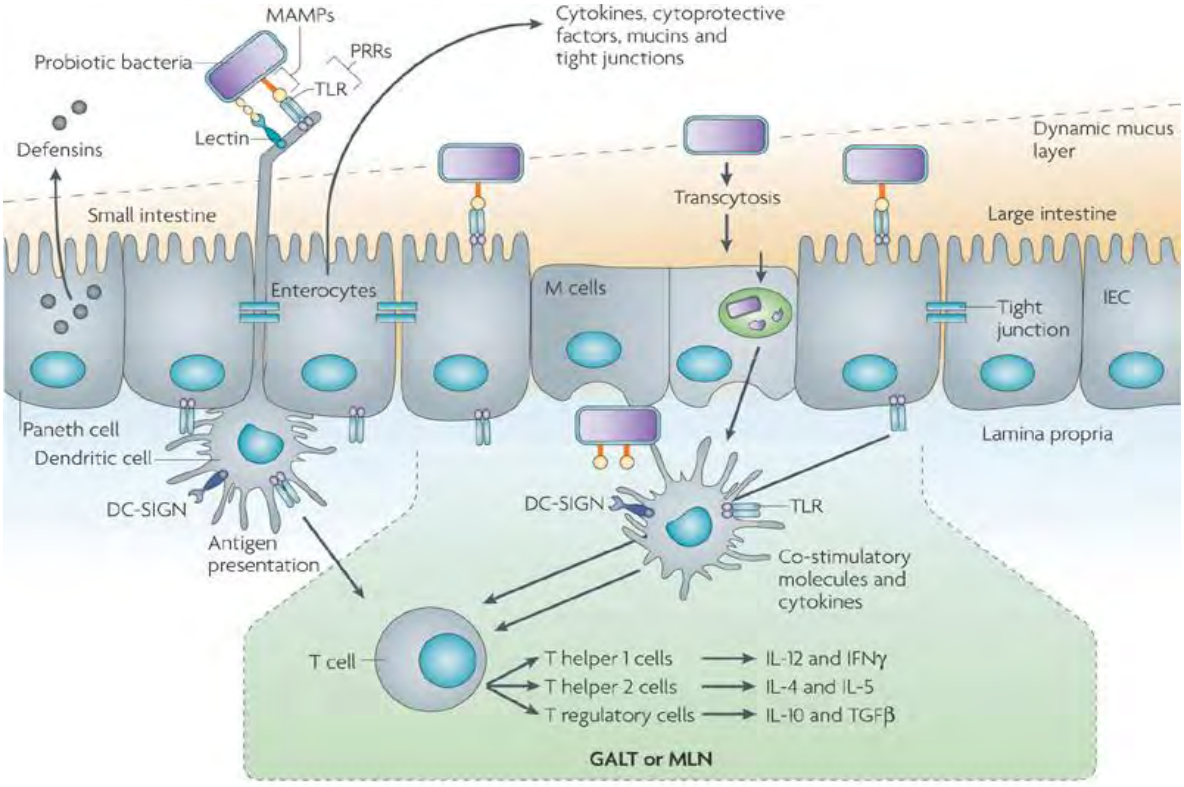

Figure 2. 10: The putative mechanism of interaction of probiotics with intestinal microbiota (Krishna Rao *et al.*, 2013)

2.3.7 Root associated safety of probiotics

In the early ages of the probiotic's era, probiotics did not need to be from human origin; if it were from human origin, it would be considered relatively safe and efficient. From an evolutionary point of view, it is not wrong to say that it is not as much as crucial for probiotics to be of human origin as they are supposed to be. The condition for probiotic to be from human host relays to the seclusion of probiotics instead of the origin. In general, the probiotics that are argued to be from a human host are isolated from the fecal samples

of healthy volunteers are then proposed to be the members of healthy normal gut microbiota. The isolation of a bacterium from feces of an individual does not mean guarantee that the bacterium is undoubtedly from the normal flora of this particular human subject. It is quite possible that microorganisms passing through the intestinal tract fleetingly can also be isolated from the fecal material. In reality, it's impossible to sort out the trustworthy source of any probiotic isolate, irrespective of whether it has been isolated from fecal, dairy-associated products or another origin. Isolation of a probiotic bacterium from fecal material of a healthy human subject is not an assurance for the safety of that probiotic bacterium; fecal sample will carry typical microorganisms including opportunistic or low-level true pathogens at the sub-clinical level. So, it is proposed that rather than focusing on the initial step of isolation, sorting out the new potent probiotics isolates should concentrate on the functional characteristics of the isolate instead of the source. As alive, probiotic isolate has to be taken in enormous amounts, over a continued duration, to have its positive impacts; the safety of these microbes is of central interest (Leroy *et al*., 2008). Till now, reports on harms associated with probiotic microbes are rare. However, numerous species belonging to the genera of *Enterococcus, Bifidobacterium, Lactobacillus, Leuconostoc,* and *Pediococcus* were often isolated from many types of infectious wounds. For example, from the infectious endocardium lesions, the species of *L. acidophilus, L. casei, L. lactis*, *L. paracasei, L. plantarum, L. rhhmanosus, L. salivaris* and *Leuconostoc mesenteroides* have been isolated. Similarly, the species of *L. rhamnosus, L. plantarum, Leuconostoc. mesenteroides, Pediococcus acidilactici, Bifidobacterium eriksonii*, and *Bifidobacterium adolescentis* have been isolated from the blood samples collected from local infection sites or from the patients with systemic bacteremia. Although slight side effects have been reported to be associated with the use of defined probiotic isolates, actual infections due to probiotics happen only in immune suppressant subjects or in those having damaged gut linings (Leroy *et al.*, 2008).

An interest linked with the utilization of probiotics is the existence of antibiotic resistance genes either on chromosomes, plasmids, or transposon among probiotic bacteria. Currently, limited data is available about the mechanisms through which these genes could spread to pathogens, and it is unrevealed if the situation could arise where this would become a critical issue (Leroy *et al*., 2008). When selecting probiotic isolates, the

WHO/FAO experts urge that probiotics should not carry any transmissible antibiotic resistance gene that makes it resistant to routinely used antibiotics (FAO/WHO, 2002). For evaluating the safety of probiotics microbes and products, WHO/FAO has framed rules, advising that probiotic isolates should be assessed for some known safety parameters, including antibiotic resistance pattern, toxicity level, metabolic and hemolytic properties, and infectivity in immune suppressant subjects (FAO/WHO, 2002). And hence the *in vitro* characterization of probiotics may include, along with others, antibiotic resistance tests, assessment of virulence factors, and resistance to hot's immune system and production of and initiation of hemolysis. Various experimental animal models, including immunodeficiency, endocarditis, colitis, and carcinoma, have been used in the safety evaluation of probiotics. In few models, even acute toxicity linked with probiotics has been tested. And finally, clinical interventions have provided sufficient information regarding the safety of probiotics for human use.

2.4 Artisanal fermented Dahi

2.4.1 Introduction

Dahi is a superior artisanal fermented dairy product from South Asian countries, specifically Bangladesh, India, and Pakistan, where it is widely consumed either a chunk of the daily food or a stimulating beverage (Khorshidian *et al.*, 2020). Dahi is prepared from sub-pasteurized cow, buffalo, or goat milk through inoculation of 2-3% undefined mixture of starter culture or junk from the previous badge of Dahi (Harun-Ur-Rashid *et al.*, 2007). Dahi fermented from buffalo milk has white color, compacted body, and somewhat grainy consistency, while Dahi fermented from cow milk is compact, firm, and smooth in consistency, while a caramelized flavor is specifically associated with artisanal fermented Dahi. In ancient time it has been used for the treatment of many digestive diseases. Hence, it is known by the general public for its therapeutic properties. Dahi contains all the nutrients of the source milk from which it is prepared but in a more digestive form. The traditional Dahi is thought-out a commercial yogurt like product. However, Dahi and yogurt are unlike concerning the flavor, type of starter culture being used, processing method, and indigenous microflora. Dahi has a pleasant flavor associated with diacetyl content, mainly produced by citrate-fermenting lactic acid bacteria. In distinction

acetaldehyde flavor is mainly associated with yogurt (Harun-Ur-Rashid *et al.*, 2007; Raynal-Ljutovac *et al.*, 2008). The distinctiveness of artisanal fermented dairy products is mostly governed by the manufacturing process (Delgado *et al.*, 2017). The native bacteria of the Dahi are mostly belonged to lactic acid bacteria, including many species of *Streptococcus, Lactococcus, Enterococcus, Lactobacillus, Pediococcus,* and *Weissella* (Harun-Ur-Rashid *et al.*, 2007; Masud *et al.*, 1991b; Mehmood *et al.*, 2009; Nawaz, 2008). Dahi is also utilized as a carrier for selective probiotics, which are incorporated in the product and based on this, several types of Dahi are prepared for the consumer's benefits, like fruity Dahi, sugared Dahi, whole-milk Dahi, and skim-milk Dahi (Delgado *et al.*, 2017).

2.4.2 Starter cultures for fermentation of artisanal Dahi

For traditionally fermented Dahi, no designed isolated starter cultures are present in the dairy market. It is being formed traditionally and thus starter cultures from the previous badges are being transferred to the next badge. Research has shown that the mix starter culture mainly consists of homo and heterofermentative lactic acid bacteria and either of these can belong to either mesophilic or thermophilic groups (Ghosh & Rajorhia, 1990; Masud *et al.*, 1991b). The most known representative members from the homofermentative starter cultures are *Lactobacillus lactis* subsp *lactis* and *Lactobacillus lactis* sub *cremoris*. The *Lactobacillus lactis* subsp *lactis* is known to release FAA (free amino acids), which are supposed to enhance the growth of *Lactobacillus lactis* subsp *cremoris*. One significant specie in the mesophilic-starter group is the homofermentative *Lactobacillus lactis* subsp. *lactis* biovar diacetylactis, is known for the production of various compounds, including diacetyl and carbon dioxide. The members of the heterofermentative group are known for aroma production in the product. *Leunostic mesenteroides* is the known representative member of this group (Leroy *et al.*, 2004).

Homo-fermentative thermophilic starter microorganisms, like *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, are utilized to get slightly sour Dahi. These starters are vital in creating a righteous class Dahi with a stable and even texture with a sweetened fragrance and sour perception (Leroy *et al.*, 2004).

2.4.3 Microbiological quality of Dahi

Traditional Dahi can generally be considered as 'hygienically safe.' Because Dahi have high acidity (0.7 – 1.0%), pathogenic bacteria like *Salmonella* spp or Coliforms will not survive in this environment (Owusu-Kwarteng *et al.*, 2020). Furthermore, even if they survive, they will not be active. In addition, the lactic acid bacteria native to the Dahi produce inhibitory compounds that can kill the pathogens. Numerous trials have established that lactic acid bacteria extracted from Dahi can yield antipathogenic peptides that are effective against many pathogens (Anal, 2019). For microbiological standards of Dahi, the highest allowable number for yeasts and mold is 100, while for coliforms it is 10 cfu/g^{-1}, defined by the BIS (Anal, 2019).

2.4.4 Microbiota of Dahi:

The microflora of Dahi mainly consists of Lactic acid bacteria species and lactose fermenting yeasts. Some common species of lactic acid bacteria that are found in Dahi are *Lactococcus, Streptococcus, Enterococcus, Leuconostoc, Weisella,* and *Lactobacillus* (Harun-Ur-Rashid *et al.*, 2007b; Harun-Ur-Rashid *et al.*, 2007; Mehmood *et al.*, 2009; Nawaz, 2008). Also, some spore formers and coliform have been associated with Dahi (Lopez-Brea *et al.*, 2018). It is believed that milk inside the udder of milking animals is sterile, which later on enriches with external microorganisms upon exposure (Panigrahi *et al.*, 2018). These microbes can come from various sources like atmosphere, herd environment, milking equipment handling, processing, and storage (Laslo & György, 2018). Due to these factors, the microflora of Dahi is highly variable, and these are the factors that determine the nature and extent of variability. Yeast containing Dahi, if kept for long at room temperature, becomes sour due to yeast activities. Hence, a high number of yeast and coliforms are considered contaminants of Dahi. Though, environment of Dahi is not favoring the growth of pathogens but some of the pathogens like *Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes,* and *Salmonella paratyphi* can survive in Dahi (Anal, 2019; Keisam *et al.*, 2019).

2.4.5 Dahi; a source for potential probiotics

According to the International Scientific Association for Pre and Probiotics (AIPAP), probiotics are live microorganisms that confer health benefit on the host when taken in an adequate amount. The most used probiotics in food and feed are from *Lactobacillus* and *Bifidobacteria,* but many other bacteria and fungi are also included. Dairy products are naturally rich in carbon sources and essential amino acids act as best growth media for many microorganisms. The buffering property associated with milk and its fat contents makes the microbes able to tolerate the harsh conditions of the gastrointestinal tract. Traditional fermented dairy products harbor a diverse microbiota comprising a variety of species of lactic acid bacteria. As these foods have been used for centuries and their safety is well known, there is a growing interest in isolating lactic acid bacteria, identifying them, characterizing them for probiotic properties, and using them for specific purposes. Several members of lactic acid bacteria have been isolated from traditional fermented milk product Dahi and characterized for probiotic properties. These members include *L. mesenteroides spp, L. bulgaricus, S. thermophilus, L. reuteri, L. planetarium, L. casei,* and *L. acidophilus.* Several Dahi origin strains have been used for specific purposes. A study showing the effect of probiotic Dahi containing *L. acidophilus* and *L. casei* against *S. enteritidis* in mice depicted that probiotic Dahi, when given to mice, boosted their innate as well as specific immunity by increasing the production of cytokines and lymphocyte proliferation and decreases inflammatory response induced by *S. enteritidis* (Jain *et al.*, 2008). The *S. enteritidis* attacks intestinal epithelium and causes an inflammatory response. It was found that inflammatory markers that are B-galactosidase and B-glucuronidase activities were increased in infections caused by S. enteritidis. After feeding probiotic Dahi to mice it was found that it improved the activities of these enzymes in the intestinal fluid and hence suppressed the inflammatory action caused by *S. enteritidis* (Jain *et al.*, 2008). The isolated strains of *L. acidophilus* and *L. casei* also displayed an antagonistic behavior against *S. enteritidis* growth (Jain *et al.*, 2008). Another study conducted by Yadav *et al.*, 2007 has that probiotic *L. acidophilus and L. casei,* isolated from Dahi, have a detaining effect on the progress of diabetes which was caused due to high fructose induction in the rats (Yadav *et al.*, 2007). The strains of *L. bulgaricus and Streptococcus thermophilus* isolated from Dahi have been characterized for probiotic potentials (Mahmood *et al.*, 2013). These strains

have a high capacity for lactose utilization and are used to treat lactose intolerance (Mahmood *et al.*, 2013). Research conducted in Bangladesh showed that *Lactobacillus paracasae* STII isolated from Dahi is beneficial for children with non-rotavirus-induced diarrhea (Harun-Ur-Rashid *et al.*, 2007b). The strains of *B. bifidum and L. acidophilus* have also been used for the treatment of colon cancer in rats. It was observed that when probiotics strains were given orally, they decreased the concentration of fecal azoreductases, nitroreductases, and B-glucorinidase enzymes, for converting procarcinogen into true carcinogens in rats (Mohania*, et al.*, 2013). In another study, it was found that glycopeptides present in the cell wall of *Lactobacillus bulgaricus* present in Dahi have antitumor activity. In an experiment, it was found that probiotic Dahi containing *L. acidophilus* and *L. casei* was fed to rats that were fed before with a diet enriched with cholesterol. The results showed that a prominent decrease in plasma cholesterol level. It was found to get as low as 22%-28% in hyperlipidemic rats. These rats were fed with a lyophilized cell of a broth culture of probiotic and probiotic Dahi. A study was conducted on the effect of Dahi containing *L. lactis biovar diacetylactis, L. delbrueckii sup bulgaris* and *S. thermophilus,* found that these strains have ability to reduce plasma triglycerol and total cholesterol in humans (Mohania *et al.*, 2013).

2.5 The *in vitro* gastrointestinal tract models to evaluate the survival of potential probiotics and their communication with intestinal microbiota

2.5.1 Introduction

The human gastrointestinal tract is tremendously an intricate and active ecosystem that play a vital role in various important functions related to the human physiology, not limited to but including absorption of nutrients, safety against attacking pathogens, and even immune development. These functions are, to some extent, done by the microbiota, that has devolved with human form the time of birth in a synergetic association (Thursby & Juge, 2017). With an inter-individual variation, the human gut microbiota is composed of approximatively 1013-14 microorganisms that make a core genome of more than hundreds times of genes compared to the human genome (Thursby & Juge, 2017). The influence of balanced microbiota on the general health of any individual host has been well-defined, while disrupted microbiota being connected to various diseases like chronic inflammation,

diabetes, inflammatory bowel disease (IBD), and obesity (Rajilic-Stojanovic *et al.*, 2020). Hence, the perception of mitigating the structure of gut microbiota is an effective approach for researchers and has directed to the utilization of some required methods in sustaining gut health, such as, through use of probiotics, prebiotics, synbiotics, and fecal microbiota transplantation (Hou *et al.*, 2020; Nogacka *et al.*, 2020; Sergeev *et al.*, 2020). Due to the integral confines in sampling from human's gut, ethical concerns, and inter and intra individual variations, efforts have been placed to expand some alternatives like *in vitro* and *ex-vivo* simulating models that mimic the human gastrointestinal tract. Although none of these models can replace -based trials, these can act as the best tools for proving conceptual and theoretical studies before human or *in-vivo trials* (Campana *et al.*, 2017).

2.5.2 A Snapshot of human GIT

The human gastrointestinal tract starts from the mouth and ends at the anus, and is divided into two main parts, the upper and lower tracts. The upper gastrointestinal tract consists of the mouth, esophagus, stomach, and small intestine. The lower gastrointestinal tract is composed of the small intestine and the colon. When the food is ingested, it is chomped and mixed in the mouth with saliva to form a bolus (Assad-Bustillos *et al.*, 2020). Besides lubricating and maintaining the physical appearance of bolus, the saliva also contains amylase enzymes that imitate hydrolyses of the starches present in the food. The pH of the saliva is primarily neutral (7 – 7.4) (Bohn *et al.*, 2018). From the mouth, the bolus enters down to the stomach (Bohn *et al.*, 2018). That is a storing bowl that regulates the delivery of food nutrients into the small intestinal tract. Initially, as food enters the stomach, the pH is comparatively high, and the enzymes are low. However, the situation quickly deviates as HCL is released and the pH decreased, and pepsinogen, the zymogen of pepsin, and gastric lipases are released into the stomach. Various enzymes existing in the bolus, now called chyme, are functional over various pH concentrations such as lipases are active at pH 5 (Bohn *et al.*, 2018), salivary amylases are active over pH 3 (Assad-Bustillos *et al.*, 2020), and pepsin is active at pH∼2. Hence their gradual activity helps in digestion and nutrient absorption (Sams *et al.*, 2016). The chyme squeezes into the small intestine from the stomach, which contain the duodenum, the jejunum, and the ileum. The chyme in the duodenum is blended with bile and pancreatin, and the pH is again nearly neutral. The

pancreatin released into the duodenum comprises various amylases, lipases, proteases, and some other enzymes. Due to comparatively high enzymatic action, the food content is quickly hydrolyzed in many products. The remaining indigestible food materials pass through the colon, where the high microbial community drives its fermentation and convert into many products. Here in the colon, many bacterial enzymes and products are present, and the pH also varies (Assad-Bustillos *et al.*, 2020). The ingested microorganism right from mouth till colon passes through fluctuated harsh conditions. The host's internal environment and capabilities of the bacteria determine where for how long these microorganisms will stay (Campana *et al.*, 2017).

Figure shows pH and salts and enzymes in different parts of the digestive system.

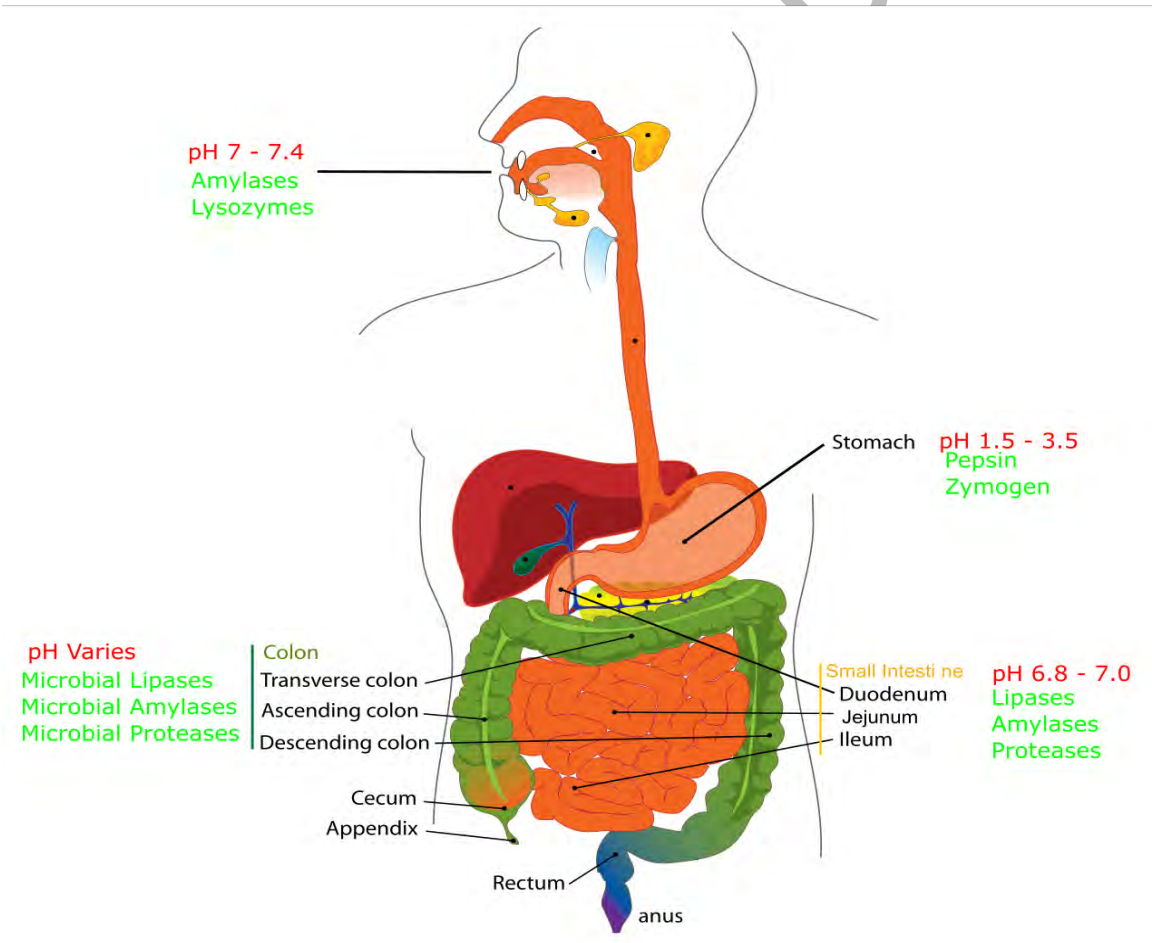

Figure 2. 11: A snapshot of the gastrointestinal tract. Different factors that affect microbial growth in the different parts of the gastrointestinal tract are illustrated in the figure.

2.5.3 The *in vitro* GIT simulation

Due to perplexity in sampling from various parts of the gastrointestinal tract, human *in-vivo* trails mainly depend on terminus information alone, typically obtained from the fecal material. Meaning that live examination of the intestinal microbes alongside the intestinal tract is not continually feasible, making it hard to test if certain types of treatment apply its impact. Also, in the existence of host-associated products, it is always hard to determine the biochemical reactions specifically associated with microorganisms (Mackie *et al.*, 2020). For these reasons, efforts were being made to find a solution that would be an alternative to the human gut and free from the problems mentioned above. Nowadays, many alternatives for the human gut are available in the market, and work is being done to make them better. Among these, the *in* vitro gut model system offers rapid, simple, and fair methods of observing the intestinal microbiota in one or several intestinal regions or down the whole intestinal tract (Bohn *et al.*, 2018). All the *in vitro* simulated intestinal models have some shortcomings, mainly associated to their weak physiochemical resemblance. These systems cannot be precisely accurate for the observations as that happens *in vivo,* because they have no epithelial mucosa, hot immune, and neuroendocrine systems (Mackie *et al.*, 2020). They do, though, allow testing the deviations in gut microbiota, concerning numbers and metabolic activities, deducible to the substratum, depressant, or illness that is directed to be measured. These models vary from single and straightforward cultures one-compartment batch fermentation to more complex single or multistage pH-regulated continuous fermentation (Bohn *et al.*, 2018).

2.5.4.1 Simulating the upper GIT

The actual human upper gastrointestinal tract consists of the mouth, pharynx, esophagus, stomach, and small intestine. However, as for the studies involving the pattern of food digestion or probiotic-related stud trials, the stomach and small intestine are often considered most important. Furthermore, hence, the physiochemical environment of the small intestine and the stomach had been focused on simulation (Mackie *et al.*, 2020). The primary and key features for simulation are body temperature, movement of saliva, gastric juice, pancreatic juice, digestive enzymes, and bile acids; peristalsis for amalgamating and intestinal transit times; control of gastric and intestinal pH; and incessant elimination of

processed lipophilic and hydrophilic wastes. The new simulating models are also able to mimic exact features related to age (e.g. infant, adult, elderly), the type of food (e.g., liquid, solid, high- or low-fat), and the wellbeing status (health or disease) (Mackie *et al.*, 2020).

2.5.4.2 Stimulating the lower GIT

The natural human lower intestinal tract contains two main parts the small intestine and the large intestine. Bacterial fermentation mainly occurs in the colon, and most models have simulated the environment of the human colon (Mackie *et al.*, 2020). The simulation includes colonic pH, mimicking the anaerobic condition of the colon, and a steady slow inflow of food predigesta coming from the small intestine. Moreover, being an essential factor, these simulating models also mimic the human gut microbiota with the same number and complexity that is has been observed in humans. Consideration has also been put on removing waste products from the compartments in a mimic human way (Mackie *et al.*, 2020).

2.5.5 Available simulating models:

2.5.5.1 Single compartment (batch fermentation)

The batch fermentation simulated models mostly contain single compartment bioreactor having basic media, augmented with test factor (substratum/suppressant), maintained at body temperature (37ºC) and anaerobic condition (Takagi *et al.*, 2016). The inoculum is quickly progressed to its stationary phase due to accumulation of toxic byproducts and nutrient depletion, resulting in a short incubation time for the experiment. For colonic fermentation this model has been used frequently (Takagi *et al.*, 2016).

2.5.5.2 Multi-compartment (continuous fermentation)

The intestinal simulated models, based on continuous fermentation, give an environment that is more similar to the conditions found in the intestinal tract, such as a continuous in-flow of food and out-flow of wastes at a constant retention time. The pH, temperature, and atmospheric conditions are also mimicked in these models. In the human colon, the initial portion (ascending part) is rich in nutrients while acidic due to microbial fermentation, which then becomes basic and nutrient low towards the middle part (transverse) and becomes neutral while nutrient-depleted at the end (descending portion) (Wang *et al.*,

2019). A three and four compartment continuous fermentation is used (Attri & Goel, 2018; Hernandez-Sanabria *et al.*, 2020). The test inoculum is added in the first-ever compartment, which then is shifted to the second and third one while wastes are collected from the last compartment. Incubation time for these simulated models is 15-16 hours (Attri & Goel, 2018; Hernandez-Sanabria *et al.*, 2020).

2.5.5.2.1 SHIME model

The human intestinal microbial ecosystem simulates five vessels simulating the stomach, small intestine, and colon. The first two vessels simulate enzymatic and physicochemical parameters like pH, retention time, inoculum size, concertation of enzymes and salts along and absorptive process of intestine and stomach. The remaining three vessels mimic the human colon. Incubation time for this system is two weeks (Duysburgh *et al.*; Oddi *et al.*, 2020).

2.5.5.2.2 TNO intestinal model

TNO intestinal model (TIM) is contained dual corresponding apparatuses, TIM-1 and TIM-2. The TIM-1 structure comprises eight units representing the stomach, duodenum, jejunum, and ileum, including peristaltic arrangements, pH regulator for every unit, enzymes discharge and movement, the quantity of bile, physical intestinal transit periods, and assimilation ability (Hoshi *et al.*, 2021). The TIM-2 system contains of four units in a circle representing the distal colon with peristaltic stirring, water, and metabolites assimilation through a muffled fiber crust (Hoshi *et al.*, 2021).

2.5.5.2.3 Immobilized mucus model

Mucin beads and porcine mucin gels have been used in various simulated models to simulate the mucus membrane present in the human gastrointestinal tract (Rodes *et al.*, 2013). This simulated mucus can be placed in any specific region of the simulated model and detached over a period of about 48 hours for formation and analysis of microbial biofilm (Williams *et al.*, 2015).

2.5.5.2.3 HMI model

The HMI model consists of dual sections divided by a purposeful binary sheet made of an upper luminal section covered with a mucus coating, and a lower semi-porous sheath (Marzorati *et al.*, 2014). Cells (Epithelial and/or others) are cultivated in the basilar section of the unit, with the semi-porous layer permitting the discharge of the metabolic products from the simulated layer. Integration of this layer decreases cytotoxicity from direct contact among the microbial community and the epithelial layer, running the trail for a few to 48 hours. Moreover, dual but separated compartments exert varied oxygen pressures on both borders of the layer to create ideal environments for aerobic cells in the bottom region and the anaerobic microbes in the luminal area. This model can be used for studying microbial metabolites, their biotransformation, or drug designing (Marzorati *et al.*, 2014). This model can be also be used in combination with the SHIME system, hence providing an extra host segment by allowing investigation of mixed microbial populations (Marzorati *et al.*, 2014).

2.5.5.2.4 Gut-on-a-Chip

The gut-on-a-chip model provides a micro tool for investigating host-microbe communications. The model contains two microfluid passages divided by a permeable (10μm) film covered with the extracellular milieu, in which growing Caco-2 cells are stretched in the form of a monolayer (Jalili-Firoozinezhad *et al.*, 2019). Constant peristaltic propelling of medium (flux rate = 30μl/h) on this cell's monolayer accelerates epithelial maturation, improve gut barrier functionalities, and induce expansion of villi-like projections. Along with this, the cells that contour the crypts between villi contain four different types of epithelial cells (i.e., absorptive, mucus-secretory, enteroendocrine, and Paneth cells), which inhabit the same places predicted in the human small intestinal tract. This platform can be used to study host-microbe interaction, intestinal physiology, drug screening and development. The incubation time for this model is up to two weeks (Jalili-Firoozinezhad *et al.*, 2019).

2.5.6 Advantages of *in vitro* simulating models.

Some advantages are associated with the use of simulated in vitro models. These models are not too labor-intensive, not expensive, quicker, and do not need the ethical constraint that *in vivo* trials require. These models are appropriate for performing automatous studies

and hypothesis testing because of their precise conditions, duplicability, and ease of sampler at the concentration site (Brodkorb *et al.*, 2019). On one hand, if suitable for predictive studies, these models may not be suitable for comparative studies until some required standardizations are not done. The *in vitro* simulating models evade inter-individual differences that are observed in human-based trials (Egger *et al.*, 2019).

2.5.7 Disadvantages of *in vitro* digestion

The implications of any object under observation are connected to local concentrations at the site of action, which in the human body is influenced by many factors like the combination of physiological, neurological, hormonal, and immunological factors (Brodkorb *et al.*, 2019). While no simulated model has been succeeded in mimicking all these conditions 16. In the case of experiments in simulating models, there is always the concern of whether the same result would be observed in humans. Another possible drawback is their ease of use that can lead to inappropriate use (Egger *et al.*, 2019).

Chapter 3
Isolation, Identification and Characterization of Probiotic Bacteria from Artisanal Fermented Milk product (Dahi)

3.1 Introduction

Dahi is a traditional fermented milk product that is the most used fermented milk-based product in Pakistan (Nawaz, 2008). It is used regularly as a part of daily meals and is also used in the formation of other cookery objects and therapeutic purposes. Dahi is traditionally prepared by adding undefined mixed starter cultures or a spoonful of leftover junk from the previous badge of Dahi to boiled or sub-pasteurized milk (Faccia *et al.*, 2020; Mudgal & Prajapati, 2017). Dahi is similar to commercial yogurt in its colour and appearance but unique in taste, texture, microbiology, nutritional and therapeutic values (Mudgal & Prajapati, 2017; Nawaz, 2008; Nawaz *et al.*, 2019a). Such as, it contains nutrients existing in the source Milk, excluding the changes triggered due to bacterial growth and fermentation (Mudgal & Prajapati, 2017). The microbiology of the Dahi is highly diverse and relies on different factors such as the atmosphere, utensils, and human hands. In addition to numerous LAB species, Dahi contains lactose utilizing yeasts, coliforms, and spore formers (Bhattarai *et al.*, 2013). The lasting quantity of lactose next to its fractional usage by LAB for making lactic acid and other composites pays to the caloricity of Dahi and can improve the detrimental effects of lactose deficiency (Mudgal & Prajapati, 2017). Dahi has been reported to contain 3-30% more proteins than the milk from which it is fermented. Due to microbial activity, the natural milk proteins are transformed into fluffy mash comprising purely isolated casein that is frequently assimilated than milk proteins (Mudgal & Prajapati, 2017; Nawaz *et al.*, 2016). Also, the free amino acids like methionine, lysine, and cysteine are produced by different microbes present in the Dahi and are beneficial for Muscle's and Liver's health. Microorganisms in the Dahi enhance free fatty acids through lipolysis of milk fat and form complex linoleic acid through internal linoleic acid, which can deliver nutritive and health effects (Mudgal & Prajapati, 2017; Robinson, 2005). Dahi has been proved to have an anti-allergic effect, anti-oxidative effect, anti-carcinogenic effect, antidiabetic effect, anti-atherogenic effect, lactose intolerance, immune enhancement, antimicrobial effect, and anti-diarrheal effect. Based on these properties, it can be said that if bacteria are extracted from Dahi and characterized it may have probiotic potentials (Eshaque, 2018).

Probiotics are live microorganisms that deliver health benefits to the host when ingested in adequate amounts. Various lactic acid bacteria (LAB) are widely marketed as

probiotics, and their proposed health-associated properties as probiotics include inhibiting pathogen's attachment to intestinal linings, halting the growth of pathogens, and maintaining intestinal integrity (Ghosh *et al.*, 2019). Further, LAB is an integrated part of numerous fermented food products, and hence isolation and identification of LAB from a variety of artisanal fermented products have become an era of intensive research during the last decades (Plaza-Diaz *et al.*, 2019). The LAB isolates, obtained from artisanal fermented products, can be applied in fermentation of various food products with desired features that possibly can inhibit pathogens *in vivo*; this would benefit inhibition and/or curement for infections in customers (Nawaz *et al.*, 2019b).

An effective probiotic candidate must be apt to bear the gastric pH with confrontation to bile salts and stick to the surfaces of epithelium. They must also deliver health-related benefits like antipathogenic activity, toxin neutralizing ability, or improving immune response. Therefore, potential probiotics should be evaluated *in vitro* erstwhile declaring them as probiotic candidates (Ghosh *et al.*, 2019; Zommiti *et al.*, 2020).

The present study was based on a critical objective to isolate potentially probiotic bacteria from fully un-explored, artisanal fermented milk product Dahi. The *in vitro* trails described the isolated candidates for probiotic characteristics that could be used for fermentation and preservation of food.

3.2 Material and methods

3.2.1 Collection of Dahi

Thirty-seven freshly prepared Dahi samples were collected from individual small-scale Dahi producers of Rawalpindi, Islamabad, Pakistan in sterile bottles and were immediately shifted to the Laboratory of Microbial Food Safety and Nutrition, department of Microbiology, Quaid-I-Azam University, Islamabad. On arrival to the laboratory, samples were subjected to pH determination and divided into two equal parts, one part was processed immediately for isolation of probiotic bacteria, and the second part was stored at -80^{o}C.

3.2.2 Isolation of the probiotic bacteria

The isolation of the probiotic bacterial isolates from Dahi samples (a traditionally fermented milk product) was grounded on the procedure used by (Nawaz *et al.*, 2019b).

Concisely, samples of Dahi were collected under clean settings in the early morning soon after completion of its designed incubation time from different corner dairy shops of Rawalpindi, Islamabad Pakistan. These vendors used Milk from different sources (Cow or Buffalo Milk) for preparation of Dahi and shifted to the laboratory for further analysis; about 1 ml in case of liquid/semi-liquid and 1 g in case of solid sample was taken as initial dilution and was serially diluted in 0.85% (w/v) normal saline (NS), plated on an MRS agar medium (Sigma, Aldrich), Skim Milk medium (SM) (HiMedia, India), and Tryptic soy medium (TS) (HiMedia, India), and then incubated at 37ºC for 24–72 hours. Morphological distinct colonies were purified into single isolated colonies via sub-culturing onto MRS, SM, and TS agar plates. The pure cultures were kept in certain media at 4ºC for further analysis. The stock cultures were preserved at -80ºC in 30% filtered-sterile glycerol.

3.2.3 Initial depiction of putative probiotic isolates

Initial identification of 143 isolates was grounded on physicochemical properties that involved Gram's staining, catalase-oxidase activity, motility, acid production, lactose utilization and curd formation (Somashekaraiah *et al.*, 2019). The isolates were checked for fermentation type (homo/hetero fermentation) and their ability to ferment different types of sugars (Data provided in Appendixes).

3.2.4 Identification of the isolates

Molecular identification of the isolates was confirmed by ON-rep-seq as described by (Krych *et al.*, 2019). Briefly, after DNA extraction through InstaGene™ Matrix (Bio-Rad Laboratories, Inc, USA), qualification and quantification, the samples were prepared for On-Rep PCR cycles. A volume of 1 µl of DNA (~20 ng/µl) was mixed with 5 µl of PCRBIO-HiFi buffer, 0.25 µl of PCRBIO-HiFi Polymerase (PCR Biosystems Ltd, London, UK), 4 µl of (GTG)5 primer and Milli-Q® water that summed a final volume of 25 µl. For PCR-1, the settings of thermocycler (SureCycler 8800, Agilent, CA, USA) were adjusted as 95°C for 5 min; 30 cycles of 95°C for 30 sec, 45°C for 1 min, and 62°C for 4 min, followed by final elongation at 72°C for 5 min.

After completion of the first cycle, 1 µl of Rep-PCR-1 product was taken and mixed with 12 µl of PCRBIO-UltraMix (PCR Biosystems Ltd, London, UK), 2 µl of repBC primers (10 µM), and 10 µl nuclease-free water. Furthermore, the thermocycler was adjusted to

settings, 3 cycles of 95°C for 30sec, 45°C for 1 min, and 62°C for 4 min, followed by 10 cycles of 95°C for 30 sec, 65°C for 1 min, and 72°C for 4 min, and final elongation at 72°C for 5 min.

After Rep-PCR-2, the concentration of DNA was measured with Qubit®dsDNA BR Assay Kit (Life Technologies, CA, USA). The quantity was recorded with Varioskan Flash Multimode Reader (Thermo Fischer Scientific, MA, USA). Fluorescence was recorded at 485/530 nm, then 10 µl of each sample was pooled into a library.

Sequencing of the prepared library was performed through MinION (Oxford Nanopore Technologies, Oxford, USA). After initial base calling, the sequenced (ON-Rep-Seq) data, de-multiplexing fastq files were taken for generation of read length count profiles (LCps) based on the sequences length dissemination. Then, reads within each peak were grouped with VSEARCH, modified with Canu, followed by taxonomy sorting with amended quality reads. Lastly, the hints were compared to guess species and strains based on LCps (Krych *et al.*, 2019).

3.2.5 Determination of potential probiotic properties of the isolates

3.2.5.1 Tolerance to bile salts and acids

Toleration of the isolated bacterial isolates to bile salts and low pH was evaluated using (Albano *et al.*, 2018) and (Choi *et al.*, 2018) with slight modification. The fresh bacterial culture was inoculated in perspective media broth, set to pH values 2, 3, and 5 with 1 N hydrochloric acid (HCl). For bile resistance, the media was supplemented with 0.25 and 0.45% oxgall. The broth adjusted to a pH of 7 with no bile salt supplementation was considered as the control. The isolates were kept incubated at 37ºC for 0, 2, and 4 hours and cultured for enumeration using respective media agar plates in duplicates. After incubation at 37ºC for 24 hours, percentage (%) survival of the isolates was determined using the equation:

$$Survival\ (\%) = \frac{CFU(T1)}{CFU(T0)} \times 100$$

CFU (Colony-forming unit), T1 (CFU at time) and T0 (CFU at time 0).

3.2.5.2 Cell surface hydrophobicity

Potential probiotic isolates' cell surface hydrophobic activity was assessed *in vitro* by measuring the microbe's grip to hydrocarbons through the assay explained by (Dlamini *et al.*, 2019). The fresh broth cultures were centrifuged for 10 mints at 8,000g and 4ºC, cleansed two times with Normal saline, and re-drooped in normal saline buffer subsequently measured its absorbance (A0) at 600 nm. A 3 ml volume of bacterial cells was combined with 1 ml xylene and kept static at 37ºC for 1 h, resulting in the appearance of two phases, organic and aqueous. The aqueous part was taken off cautiously and recorded its absorbance (A1) at 600nm. The hydrophobicity percentage was recorded by a reduction in absorbance and measured with the equation:

$$CSH(\%) = 1 - \frac{A1}{A0} \times 100$$

3.2.5.3 Auto-aggregation

The capability of the potential probiotic isolates to auto-aggregate was measured as per (Dlamini *et al.*, 2019). Fresh bacterial cultures were centrifugated for 10 min at 8,000 × g and 4ºC for 10 min), twice cleansed with saline, and re-suspended in saline. The sample was left to stand for a moment, incubating at 37ºC, and the aqueous layer was taken and measured for absorbance at 600 nm after 1 h. The percent auto-aggregation was calculated with the formula.

$$AAG(\%) = 1 - \frac{AT}{A0} \times 100$$

Where AT shows the absorbance at a time and A0 show the absorbance at time 0.

3.2.5.4 Exopolysaccharide production

For evaluating exopolysaccharide (EPS) production ability, as described by (Cerning, 1995), a modified agar medium with 10% lactose was prepared. Fresh cultures of the potential probiotic bacterial isolates were streaked on the plates and incubated at 37ºC for 24 hours. After incubation, the plates were observed for EPS production. Colonies that appeared mucoid were considered as EPS-producing isolates.

3.2.5.5 Survival during exposure to *in vitro* simulated gastrointestinal fluids

The survival of potential probiotic isolates in the *in vitro* intestinal fluids was assessed as described by (Mackie *et al.*, 2020) with slight modification. A 70 ml stock solution of simulated salivary fluids (SSF) was prepared to contain KCl 40.28 ml, K_2HPO_4 9.87 ml, $NaHCO_3$ 18.14 ml, $MgCl_2(H_2O_6)$ 1.33 ml, $(NH_4)_2CO_3$ 0.160 ml to which α-amylases (0.003g L^{-1}) Sigma Aldrich 1.375 ml, $CaCl_2$ (0.3M) 0.55 ml and sterile distilled water 31.755 ml were added. The pH of the fluid was adjusted to 6.8.

A 70 ml stock solution of the simulated gastric fluid (SGF) was prepared consisting of KCl 5.52 ml, K_2HPO_4 0.14 ml, $NaHCO_3$ 17.50 ml, NaCl 41.06 ml, $MgCl_2(H_2O_6)$ 0.56 ml, $(NH_4)_2CO_3$ 0.70 ml to which pepsin (Sigma Aldrich) 23.46 ml, $CaCl_2$ (0.3M) 0.073 ml and sterile distilled water 13.20 ml were added. The pH of the fluid was adjusted to 1.5 using 1N HCl.

A 70 ml stock solution of simulated intestinal fluid (SIF) was prepared containing KCl 9.67 ml, K_2HPO_4 0.140 ml, $NaHCO_3$ 17.54 ml, NaCl 41.38 ml, $MgCl_2(H_2O_6)$ 0.56 ml, $(NH_4)_2CO_3$ 0.71 ml to which pancreatin (Sigma Aldrich) 18.19 ml, $CaCl_2$ (0.3M) 0.146 ml and sterile distilled water 21.664 ml were added. The pH of the fluid was adjusted to 7 using 1N NaOH.

Then 1.3 ml of the simulated fluid was taken to which 0.2 ml of bacterial isolates twice washed with PBS were added. The samples were then incubated at 37ºC for 4 hours. 100µl of the samples was taken at 0-, 2-, and 4-hours intervals, and were plated onto the agar plates, incubated at 37ºC for 24 hours. The survival rate was calculated using a formula and presented as a percentage.

$$Survival\ (\%) = \frac{CFU(T1)}{CFU(T0)} \times 100$$

Where T1 is sample at time, T0 is time 0.

3.2.5.6 Evaluation of antibacterial properties

The antibacterial effect of potential probiotic isolates was assessed, through measurement of optical density, against *Bacillus subtilis* subsp. subtilis ATCC 19659, *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. enterica ATCC 27870, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. aureus ATCC 6538 and

Streptococcus pneumoniae ATCC 49619. The fresh overnight culture of bacterial isolates was initially centrifugated (5000 g, 10 min, 4ºC), the supernatant was filtered with 0.2 mm syringe filter and divided into two equal parts. One part was used as it was extracted through centrifugation i.e., the cell-free supernatant (CFS). While for the second part, to remove the assumed outcome of organic acids present in the filtrate, the pH of the filtrate was adjusted to 6.5 using NaOH/HCl (n-CFS), and both the parts were stored at -20ºC for further scrutiny.

Then a sterilized 96-well plate was poured with 50 µl of pre-prepared filtrate, 50 µl of the respective pathogen, and 200 µl of Luria Broth (Sigma Aldrich) to uphold a final concentration of 10^8 cells/well. The LB broth inoculated with pathogenic suspension was used as a positive control, and the LB broth deprived of any inoculation was used as a negative control. The well-plate was incubated at 37ºC for 24 hours, and the optical density (OD) was taken at 600 nm. The total percent pathogen inhibition was determined with the following equation:

$$Growth\ inhibition\ (\%) = \frac{OD\ control - OD\ test\ sample}{OD\ control} \times 100$$ (Georgieva *et al.*, 2015).

3.2.5.7 Evaluation of anti-fungal activity

The antagonistic effect of the potential probiotic isolates was evaluated, using agar overlay procedure, against *Candida albicans* ATCC 90028, *Aspergillus flavus* (KPO36602.1), and *Geotrichum candidum* QAUGC01 (PRJNA523005). On agar plates, potential probiotic isolates were line-streaked at equal distances and incubated at 37ºC for 24 hours. Then, 20 µl (10^6 spores/ml) spores of test fungus were consistently blended with 0.75% Potato Dextrose Agar (PDA) (HiMedia, India) and shrouded on the pre-streaked plates. After keeping at 28ºC till 4 days, the plates were checked for inhibition through observing zones around the bacterial colonies, and the results were noted either positive or negative (Somashekaraiah *et al.*, 2019).

3.2.5.8 Antibiotic sensitivity

Antibiotic susceptibility of the isolates to antibiotics was determined through disc diffusion assay using antibiotics as recommended by European Food Safety Authority (EFSA) rules (Ren, 2012). A volume of 100 µl overnight culture of the isolates was evenly spreaded on

Muller Hinton (Sigma, Aldrich) agar media plates, and antibiotic-containing discs were placed on top, and the plates were kept at 37ºC for 48 hours. The susceptibility aspect was assessed using ampicillin (10 µg/disc), gentamicin (10 µg/disc), chloramphenicol (30 µg/disc), neomycin (2 µg/disc), and tetracycline (30 µg/disc) (Suhonen, 2019). All the antibiotic discs were purchased from Sigma. The diameters of the inhibitory zones were noted and compared with the Clinical & Laboratory Standards Institute (CLSI) scale. The results attained are expressed as susceptible, moderately susceptible, or resistant. The outcomes were related through the explanatory zone distances as defined in Performance Standards for Antimicrobial Disc Susceptibility Tests (CLSI, 2020).

3.2.5.9 Bile salt hydrolase activity

A method described by (Tanaka *et al.*, 1999) was used to detect bile salt hydrolase activity of the bacterial isolates. The MRS and M17 agar media were supplemented with 0.5% (w/v) Bile salts of taurodeoxychlic acid and Glyco-deoxycholic acid (Merk, USA) 0.37g/L of $CaCl_2$. Overnight culture of the bacterial isolates was spotted on the plates and incubated at 37ºC for 24-72 hours. Precipitation below and around the colonies indicated positive bile salt hydrolase activity of the isolates.

3.2.5.10 The *In vitro* cholesterol assimilating activity of the isolates

For assessment of the *in vitro* cholesterol assimilation ability of the isolates, method described by (Madeeha *et al.*, 2016) was used with minor modification. A stock solution of the cholesterol and its dilutions were prepared. Solutions of 0.1 mg/ml to 5 mg/ml chloroform/cholesterol concentrations were prepared. From these, 0.1 ml was taken and was added to 9.9 ml of the $FeCl_3$ – acetic acid solution. Then were vortex and left for 15 minutes. Five ml of this solution was taken, and 3 ml of H_2SO_4 was added to it and vortex for 30 sec. the tubes were left for 30 min, and OD was taken at 560 nm and from this a standard curve was made for known concentrations (Given in Appendix).

The potential probiotic isolates were grown in respective broth media for 15 hours, and 100 µl of the bacterial suspension was then transferred to 9.9 ml of fresh broth. The 0.4 mg /ml (0.1ml) of this solution was added into the test tube to make available 0.04 mg/ml to the isolates and were incubated at 37ºC for 24 hours. After incubation, 0.1 ml sample was

taken from each test tube and added to the test tube having 9.9 ml of $FeCl_3$-acetic acid solution. The tubes were vortexed for 15 min, subsequently centrifuged at 8000 × g for 10 min. After centrifugation, 5 ml of the supernatant was taken in a separate sterile test tube and 3 ml of H_2SO_4 was added into it and vortexed for 30 s. OD of the sample was measured at 560 nm after 30 min of incubation in the dark.

The cholesterol assimilation was measured with the following formula.

$$Cholesterol\ assimilated = (Initial\ Cholesterol\ conc - Final\ cholesterol\ conc)$$

$$Cholesterol\ assimilated\ (\%) = \frac{Cholesterol\ assimilated}{Total\ cholesterol\ conc\ in\ media} \times 100$$

3.2.5.11 Anti-oxidant activity

Anti oxidation activty of the potential probiotic isolates was performed following the method described by (Yang *et al.*, 2020). To determine the antioxidant ability of the isolates, 2 mL of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution (0.4 mM) in methanol and 2 mL of bacterial suspension were mixed in a sterile microtube and incubated at 37ºC for 30 min in the dark. The Ascorbic acid (1 mg/ml) was utilized as a positive control. After 30 min of incubation, the tubes were centrifuged for 10 min at 8000 × g and 4ºC. The antioxidant activity was determined by recording the absorbencies of the supernatant at 517 nm and then using the following formula:

$$Antioxidant\ activiyt\ (\%) = 1 - \frac{A.sample}{A.control} \times 100$$

Where A.sample and A.control represent the bacterial sample's absorbance values and distilled water at 30 min, respectively.

3.2.6 Safety assessment of the isolates

3.2.6.1 Hemolytic activity

The hemolysis pattern of the potential probiotic isolates was observed through the method used by (Li *et al.*, 2020). The isolates were streaked onto agar plates of blood medium and kept at 37ºC for 48 hours. After incubation, the plates were observed for α, β, and γ hemolysis.

3.2.6.2 DNase activity

For assessing the DNase activity, the method described by (Almeida *et al.*, 2015) with slight modification was followed. The isolates were streaked onto DNase Medium agar plates to examine the release of DNases. Plates were incubated at 37ºC for 48 hours. After incubation, the plates were flooded with 0.1% toluidine blue and left for 5 min. The DNase positive isolates had a clear zone around the colonies streak line, while DNase negative isolates had no zone.

3.2.6.3 Decarboxylase activity

For evaluating the decarboxylase activity of potential probiotic isolates, the method used by (Bover-Cid & Holzapfel, 1999) with minor modification was followed. Briefly, broth media were prepared according to the manufacturer guidelines, and to it 0.5% tyrosine, histamine, putrescine, and arginine individually were added along with Bromocresol purple as an indicator. Then 100 µl fresh culture of the isolates was transferred to the medium and incubated at 37ºC for 24 hours. After incubation, color change was observed as positive results while no color change was considered negative.

3.2.7 Technological characterization of isolates

3.2.7.1 The enzymatic potential of the strains

The method described by (Lee *et al.*, 2001) was used to assess amylolytic activity. Surface dried plates of starch media were streaked with a fresh culture of the isolates and incubated at 37°C for 48 hours. These plates were then flooded with gram's iodine for 15 to 30 min. A clear zone around the streak lines was observed for cellulolytic isolates.

According to (Giori and Hébert, 2001), milk agar plates were prepared. Plates of milk agar were streaked with potential probiotic isolates and incubated at 37°C for 48 hours. After incubation, clear zone around the streak lines was observed for positive results.

As described by (Leuschner *et al.*, 1997), Tween 80 was used as a lipid substrate in the medium for detection of lipolytic activity of the isolates. Methyl red was added as an indicator in the media before pouring into the plates. The bacterial isolates were streaked on the lipid media plates and incubated at 37ºC for 24 hours. Change from red to yellow color was observed for lipolytic activity.

Carboxymethyl cellulose (CMC) media was prepared according to the protocol used by (Hankin & Anagnostakis, 1977). Bacterial isolates were inoculated as points on the plates and was incubated at 37ºC up to 5 days. The plates were stained with 1% Congo red and rinsed with NaCl. The Zones that appeared around the colonies were considered positive results.

3.2.7.2 Survival of the isolates at a different salt concentration

The survival of the isolates at different salt concentrations like 4 and 6% for *Lactobacillus* species and 2 and 4% *Lactococcus* species was evaluated for 2 and 4 hours at 37ºC. The viability of the isolates was assessed through the plate count method (cfu/ml), and the results are recorded as a percentage (Somashekaraiah *et al.*, 2019).

3.2.7.3 Survival of the isolates at different temperatures

The growth of the isolates was checked at a low temperature of 15ºC, and high temperature of 45ºC The growth of the isolates was evaluated by plate count assay (cfu/ml) and the results were displayed as $\log_{10}$ (Somashekaraiah *et al.*, 2019).

3.3 Results

3.3.1 Primary characterization of the isolates

Initially 143 distinct bacterial isolates were isolated from 37 different Dahi samples and were subjected to morphological, physiological, and biochemical testing as well as molecular biology-based identification using ON-Rep-Seq technique. Out of these, 55 isolates were gram negative which were excluded from further characterization, 18 Gram-positive isolates were catalase positive which were also excluded from further characterization. Seventy Gram positive, immotile, catalase-oxidase negative isolates were acids producers and lactose fermenters. Among these, 62 isolates were able to form curd from the milk. Using the ON-Rep-Seq identification method, 62 isolates were identified as lactic acid bacteria and 8 as *Bacillus* species (Table 3.1 and Figure 3.1). These 70 isolates were subjected to the characterization of desired probiotic traits, e.g., resistance to low pH, bile salts, survival in the *in vitro* simulated fluids, antipathogenic potential, sensitivity to EFSA recommended antibiotics, cell surface hydrophobicity, auto-aggregation, bile salt hydrolase activity, and cholesterol assimilating potential, 22 isolates proved to be potential

probiotic. The data of 22 strains is presented in this chapter. Fermentation mode and the ability of these isolates to ferment different sugars are presented in **Appendix.**

Table 3. 1: Number and types of bacteria isolated from Dahi samples that were selected for probiotic characterization based on ON-rep-seq identification.

Species	No of isolates	Media used for isolation
Lactococcus lactis	3	M17
Streptococcus thermophilus	2	M17
Enterococcus faecium	17	Skim Milk, M17
Enterococcus lactis	2	Skim Milk
Limosilactobacillus fermentum	23	MRS
Lactobacillus delbrueckii	2	MRS
Lacticaseibacillus rhamnosus	1	MRS
Lactiplantibacillus plantarum	2	MRS
Lactobacillus acidophilus	2	MRS
Lacticaseibacillus casae	2	MRS
Lacticaseibacillus paracasae	2	MRS
Lactobacillus gasseri	2	MRS
Limosilactobacillus reutori	2	MRS
Bacillus mycoides	3	TSA
Bacillus licheniformis	4	TSA
Bacillus subtilis	1	TSA

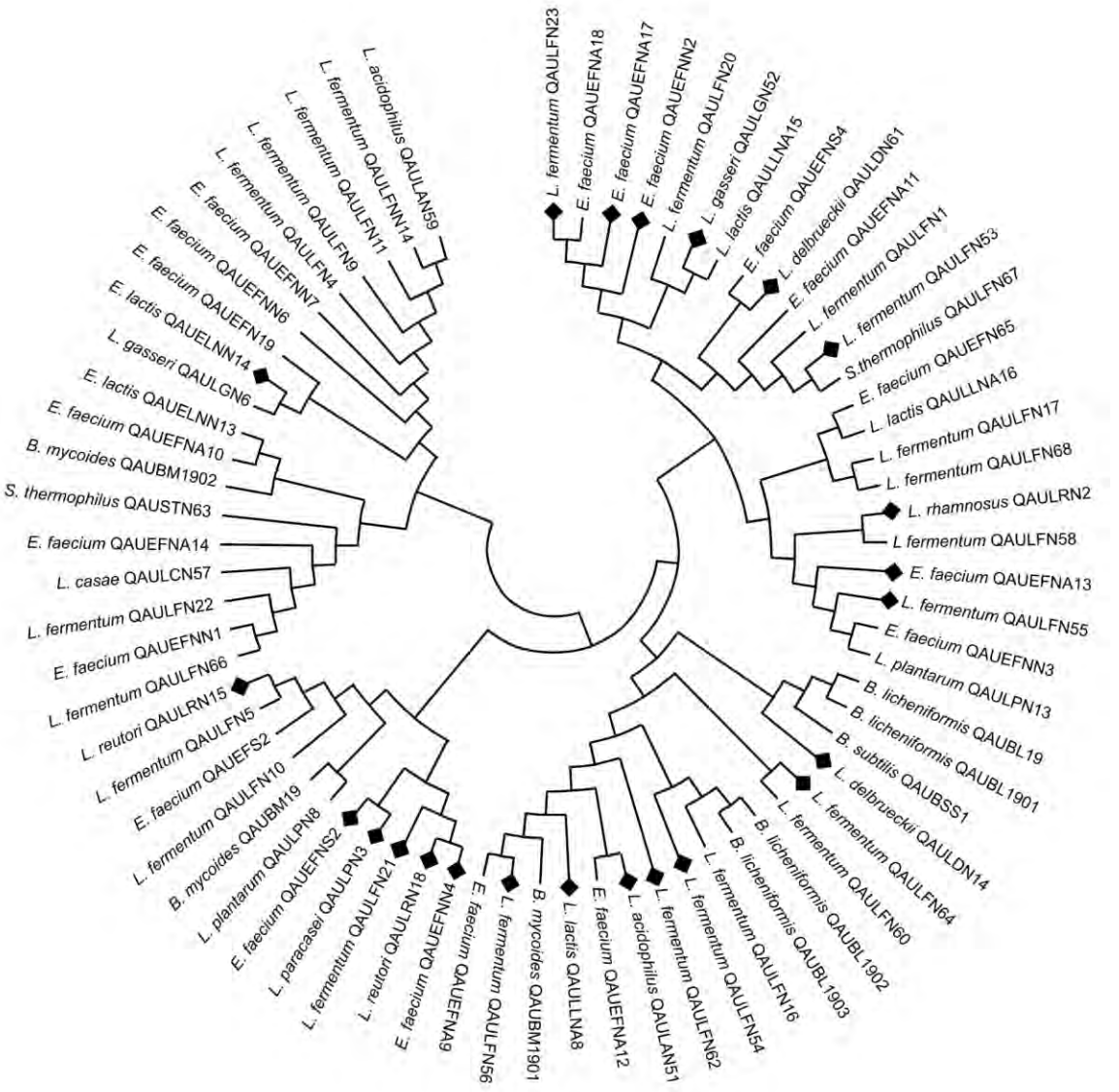

Figure 3. 1: Phylogenetic tree of all the bacterial candidates isolated from Dahi samples that were subjected to probiotic characterization (strains with best probiotic properties are highlighted with black square shapes). Rep-Seq data was used and neighbor joining method with default parameters was used for constructing the phylogenetic tree.

3.3.2 The *in vitro* determination of potential probiotic properties

3.3.2.1 Acid and bile tolerance

The ability of the bacterial isolates to survive in acidic conditions helps in their evaluation for resistance to the low pH of the stomach. The capability of the isolates to sustain low pH after 2 and 4 hours at 37ºC of incubation is given in Figure 3.2 and 3.2. Seventy

bacterial isolates were exposed to low pH and among these, only 56 isolates were able to tolerate the low pH. Among the tested isolates, *L. fermentum* QAULFN51 and *L. fermentum* QAULN51 had a 100% growth rate after 4 hours of exposure to the acidic pH while *E. faecium* QAUEFNS1 had lowest survival percentage. All the 56 isolates were able to tolerate pH 3 when were exposed for 4 hours. When exposed to pH 3, the *E. faecium* QAUEFNA12 had lowest survival rate of 50% while the *L. plantarum* QAULPN8 had highest survival percentage of 93%.

Similarly, survival in the bile salts helps in the *in vitro* survival characterization of the isolates in the gastrointestinal tract. The growth rate of isolates in bile acids after 2 and 4 hours at 37ºC is given in Figure 3.4 and 3.5. Fifty-six isolates that tolerated low pH were exposed to 0.25% and 0.45% (w/v) bile salts for 2 and 4 hours. In 0.25% bile salts, among the 56 isolates only 51 were able show more than 50% survival after 4 hours of exposures. Among the evaluated isolates, *L. fermentum* QAULFN55 had 50% of survival rate while *E. faecium* QAUEFNN3, *E. faecium* QAUNS3 and *L. fermentum* QAUEFN17 presented highest survival rate of 98% after 4 hours of exposure. These isolates when exposed to 0.45% bile salts only 49 isolates were able to show more than 50% of survival after 4 hours of exposure. The *L. lactis* QAULLNA16 showed lowest survival rate of 51% after 4 hours of exposure while *B. licheniformis* QAUBL19 and *E. faecium* QAUEFNA13 presented 100% of survival after 4 hours of exposure to 0.45% of bile salts.

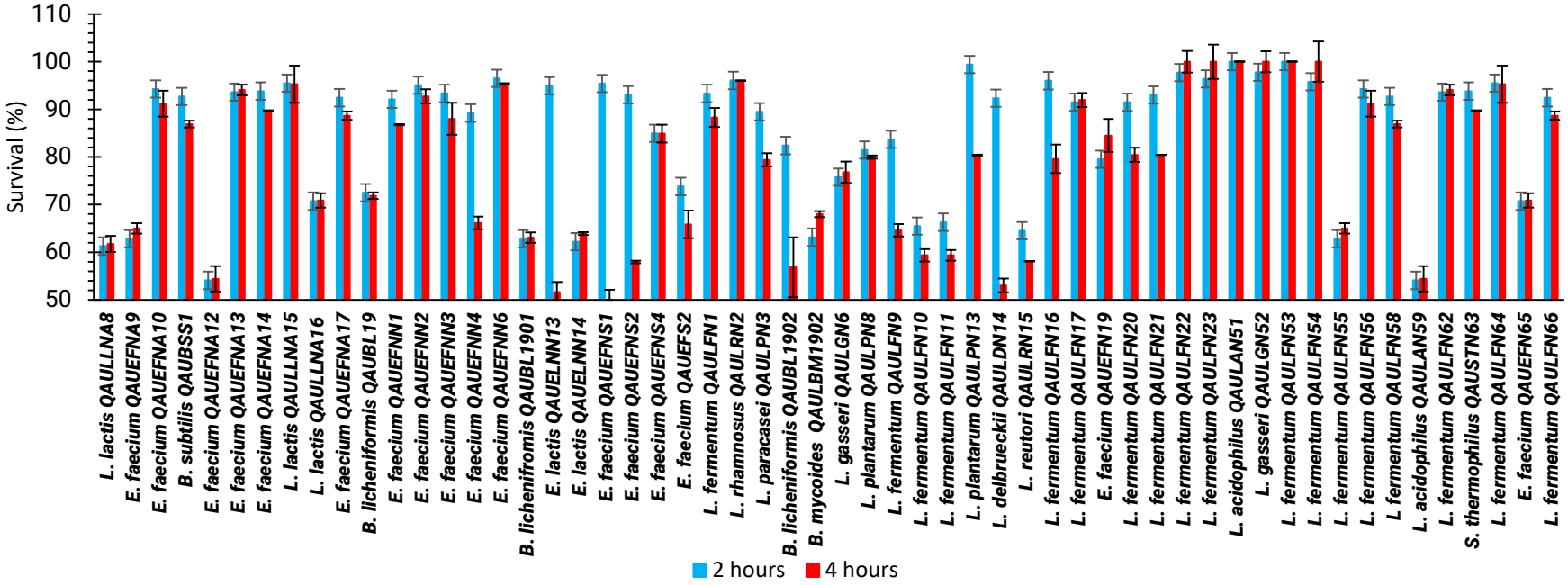

Figure 3. 2: Percentage survival of potential probiotic bacteria isolated from Dahi samples in pH 2 after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

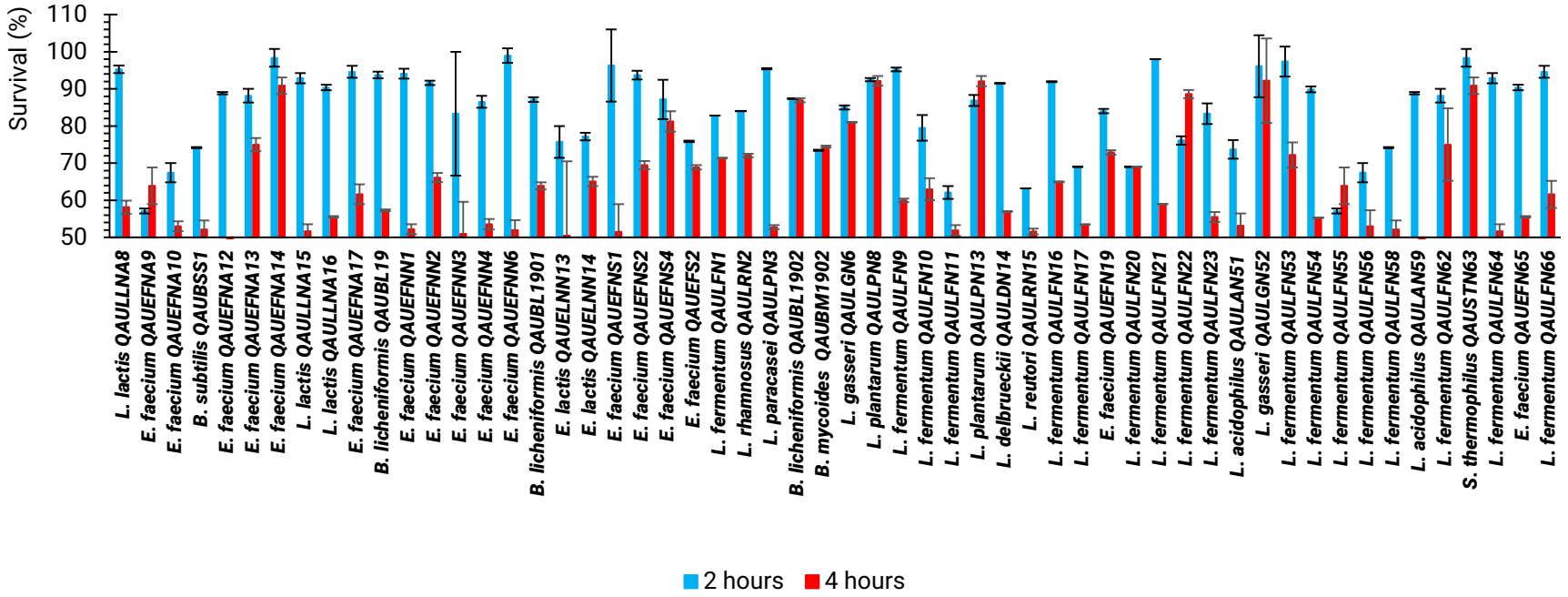

Figure 3. 3: Percentage survival of potential probiotic bacteria isolated from Dahi samples in pH 3 after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

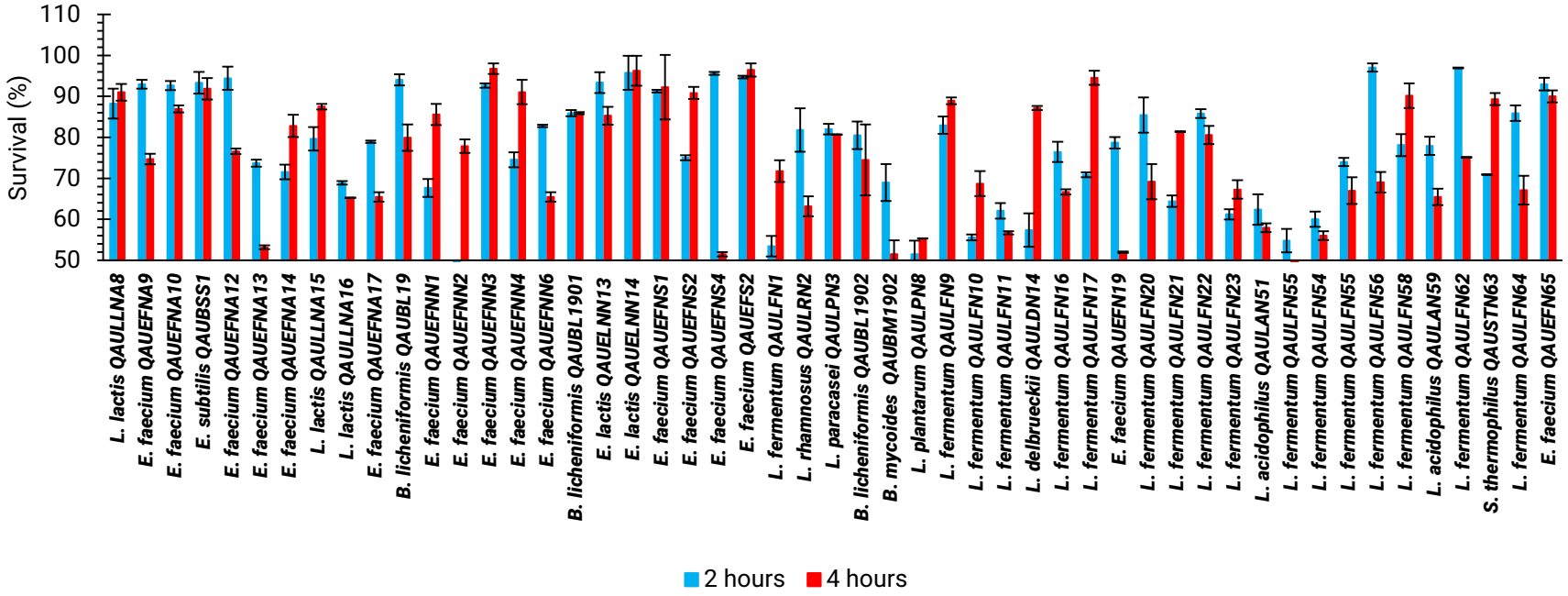

Figure 3. 4: Percentage survival of potential probiotic bacteria isolated from Dahi samples in 0.25% bile salts after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

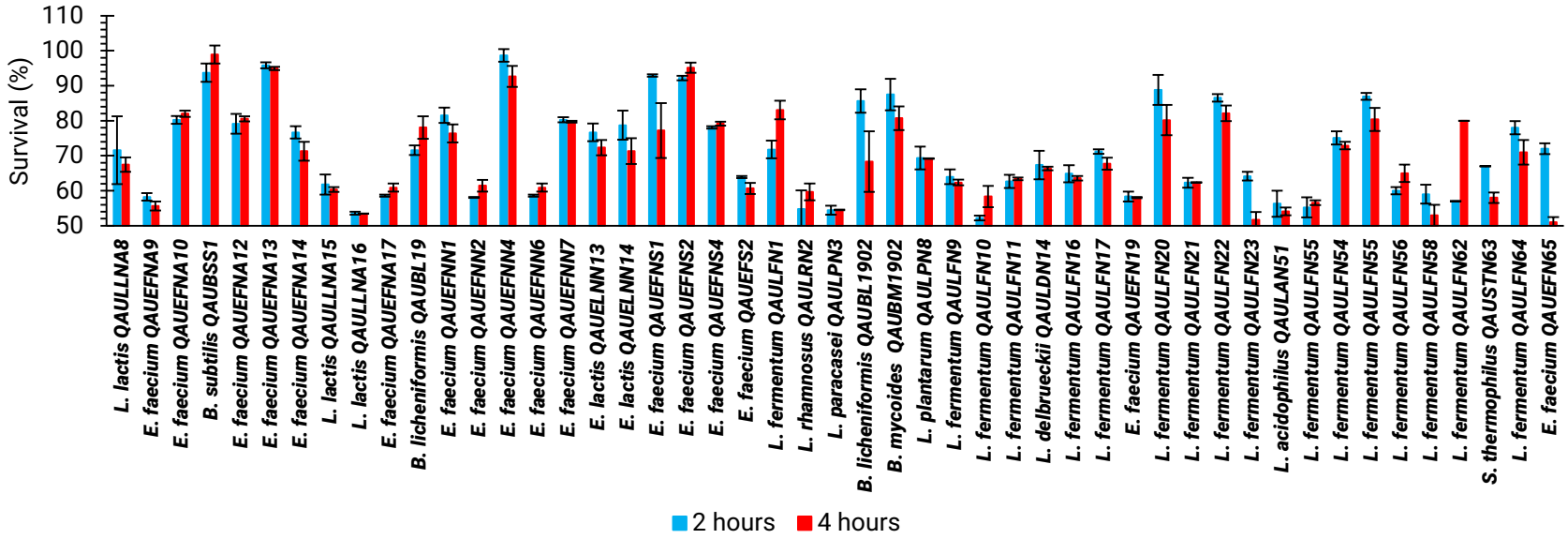

Figure 3. 5: Percentage survival of potential probiotic bacteria isolated from Dahi samples in 0.45% bile salts after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

3.3.2.2 Cell surface hydrophobicity and auto-aggregation

Forty-nine isolates that tolerated the low pH (pH = 2) and high bile salts (0.45%) were evaluated for cell surface hydrophobicity (CSH) to estimate the *in vitro* adhesivity through xylene hydrocarbon affinity. The isolates presented diverse hydrophobicities like *E. lactis* QAUELNN14 showed minimum hydrophobicity (52%) while *L. rhamnosus* QAULRN2 showed maximum hydrophobicity (99%). The isolates were checked for Auto-aggregation ability that ranged from 52% for *L. fermentum* QAULFN56 to 95% for *L. lactis* QAULLNA8. Detailed results of cell surface hydrophobicity and auto-aggregation are presented in Figure 3.6.

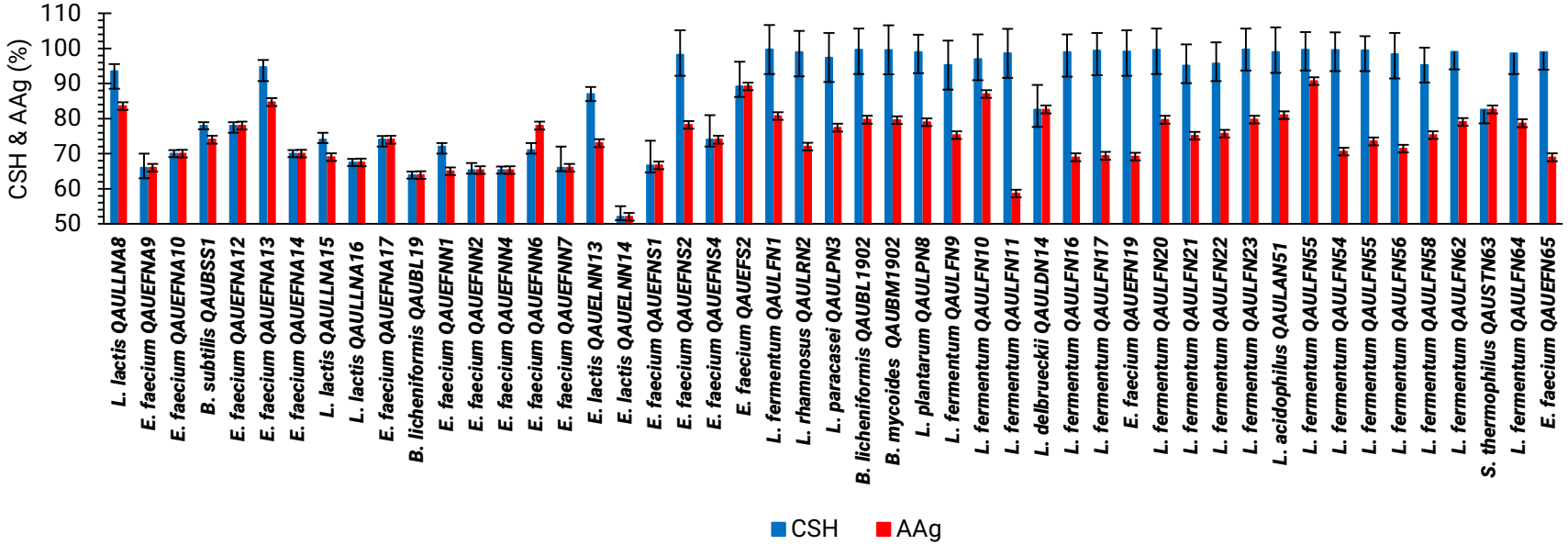

Figure 3. 6: The *in vitro* cell surface hydrophobicity and auto-aggregating abilites of potential probiotic bacteria isolated from Dahi samples. Data presented are Mean ± SD of duplicated values obtained from independent trials.

3.3.2.3 Survival under the *in vitro* simulated fluids

The isolates that tolerated low pH, high bile salts and presented cell surface hydrophobicity along with auto-aggregation were exposed to simulated gastrointestinal fluids comprising of simulated salivary, gastric, and small intestinal fluids for 2 and 4 hours at 37°C. The data is presented in Figure 3.7, 3.8, and 3.9 respectively. All the tested isolates were able to survive in the simulated salivary fluids. Their survival rate ranged from a minimum of 52% for *B. licheniformis* QAUBL1901 to 100%. When exposed to gastric fluids for 2 to 4 hours, only 22 among the 49 isolates were able to show survival percentage more than 50%. The lowest survival percentage was observed as 52% for *L. delbrueckii* QAULDN14 and highest was observed as 98% for *L. paracasae* QAULPN3. These isolates were then exposed to simulated small intestinal fluids and all the isolates were able to show survival percentage more than 50%.

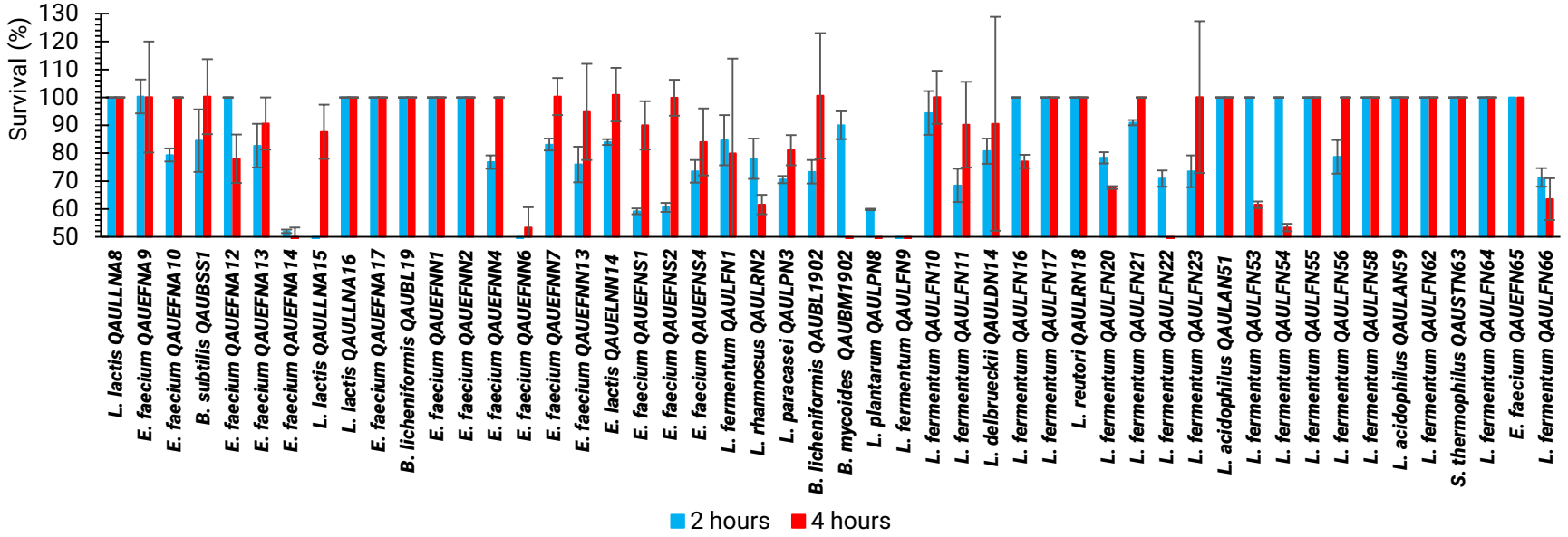

Figure 3. 7: Percentage survival of potential probiotic bacteria isolated from Dahi samples in simulated salivary fluid after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

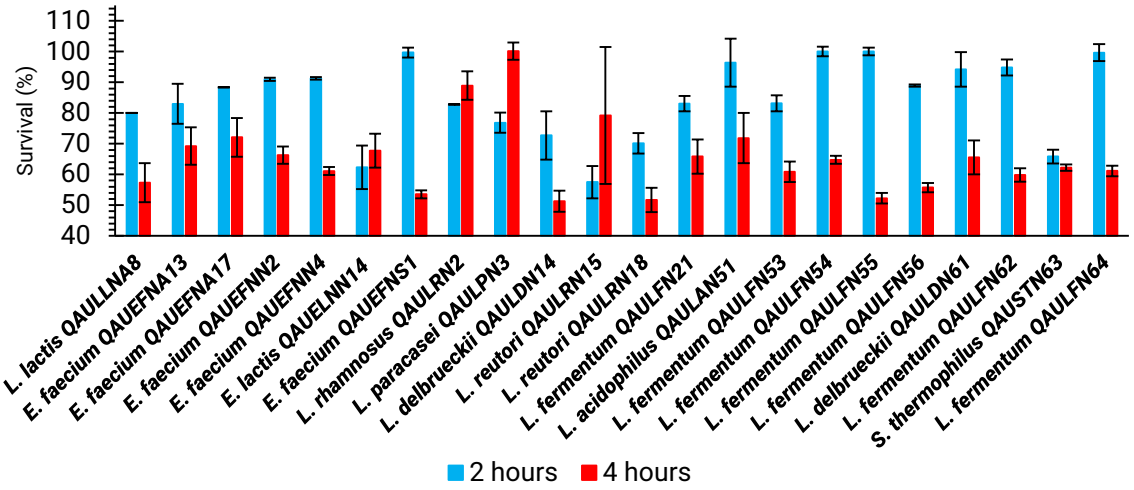

Figure 3. 8: Percentage survival of potential probiotic bacteria isolated from Dahi samples in simulated gastric fluids after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

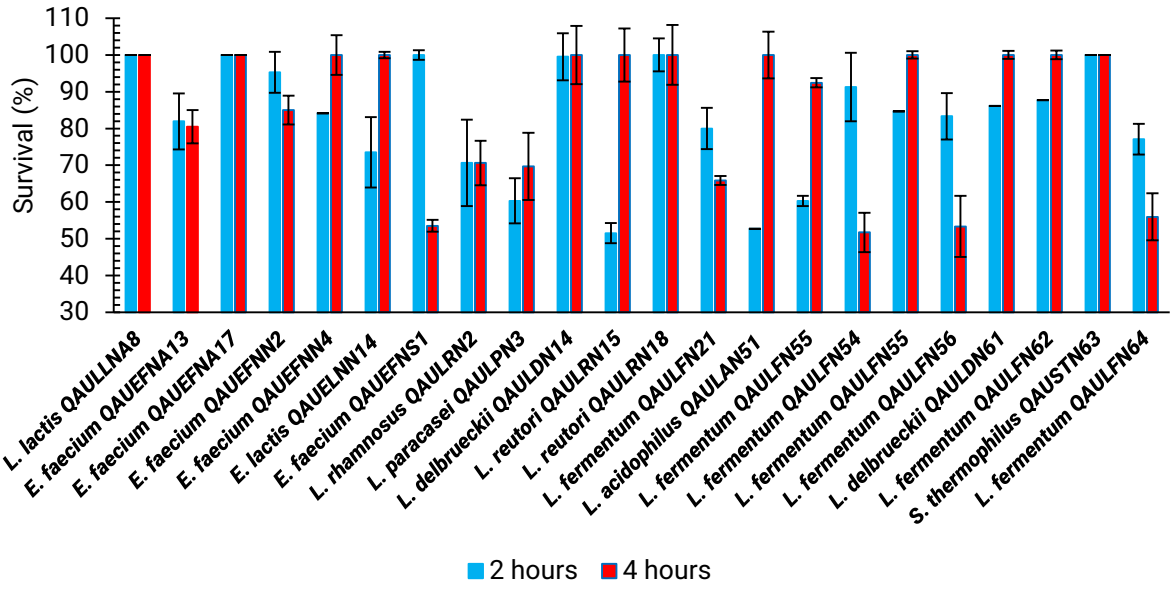

Figure 3. 9: Percentage survival of potential probiotic bacteria isolated from Dahi samples in simulated small intestinal fluids after exposure of 2 and 4 hours at 37°C. Data presented are Mean ± SD of duplicated values obtained from independent trials.

3.3.2.4 Antimicrobial activity of the isolates

The inhibitory activity of the potential probiotic bacteria against some food-borne and enteric pathogens was evaluated. The isolates proved to have substantial antipathogenic activity Figure 3.10**.** A high antagonistic effect against *Bacillus subtilis* subsp. subtilis ATCC 19659 was observed as (85%) by *L. delbrueckii* QAULDN61, and the lowest was observed as (66%) by *E. faecium* QAUEFNA13. Maximum antagonist effect against *Escherichia coli* ATCC 25922 was observed as (83%) by the *E. faecium* QAUEFNA13 and *L. acidophilus* QAULAN51 while minimum inhibition was observed as 60% by the strains of *L. lactis* QAULLNA8 and *S. thermophilus* QAUSTN63. The growth of *Salmonella enterica* subsp. enterica ATCC 27870 was affected to a range of 80% by *L. paracasae* QAULPN3 and *L. fermentum* QAULFN21. Maximum inhibitory effect against *Pseudomonas aeruginosa* ATCC 27853, was 88% by the strains of *L. acidophilus* QAULAN51 and *L. fermentum* QAULFN64 while the minimum inhibitory effect was 53% by *E. faecium* QAUEFNN2. The growth of *Staphylococcus aureus* subsp. aureus ATCC 6538 was highly inhibited by the strain *L. fermentum* QAULFN56 at 88 percent. The growth of *Streptococcus pneumoniae* ATCC 49619 was inhibited by all the isolates, but high activity was recorded as 88% for *L. fermentum* QAULFN21 and *L. acidophilus* QAULAN51, while the lowest activity was recorded as 51% by *L. lactis* QAULLNA8.

The inhibitory activity of the nCFS exhibited nominal activity toward the tested pathogens. Verifying the importance of organic acids in the antagonistic effect of these isolates.

Antifungal activity was tested against *G. candidum* QAUGC01, *A. flavus* and *Candida albican* ATCC 90028. The strains of *L. delbrueckii* QAULDN14, *L. acidophilus* QAULA51 and *L. fermentum* QAULF53 presented strong activity against *G. candidum* QAUGC01. While *L. lactis* QAULLNA8, *L. reutori* QAULRN15 and *L. fermentum* QAULFN55 presented moderate activity against *G. candidum* QAUGC01.

The strains of *L. lactis* QAULLNA8, *E. faecium* QAUEFNN3, *E. faecium* QAUEFNN4, *L. delbrueckii* QAULDN14, *L. reutori* QAULRN15, *L. reutori* QAULRN18 and *L. fermentum* QAULFN54 presented strong activity against *A. flavus*. The strains *E. faecium*

QAUEFNA17 and *L. fermentum* QAULFN56 presented moderate activity against *A. flavus.*

No activity was observed against *C. albican* ATCC 90028.

The detail anti-fungal activity of potential probiotics isolated from Dahi samples is presented in Figure 3.11.

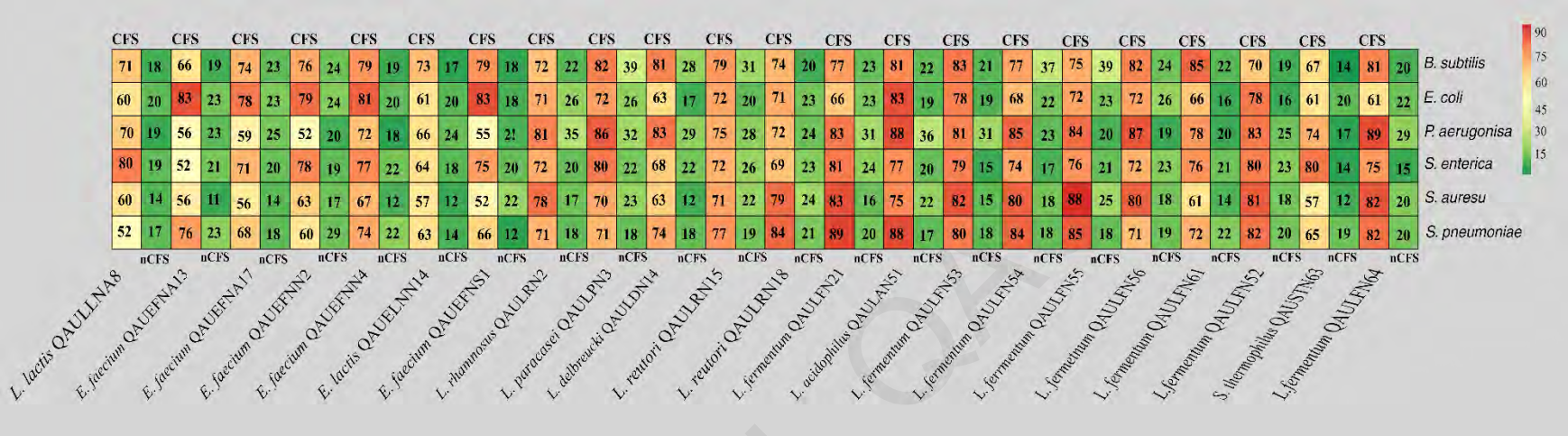

Figure 3. 10: The antibacterial effect of probiotic bacteria isolated from Dahi samples after 24 hours of incubation at 37°C (against *Bacillus subtilis* subsp. subtilis ATCC 19659, *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. enterica ATCC 27870, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. aureus ATCC 6538 and *Streptococcus pneumoniae* ATCC 49619). The data is in percentages and is presented by the color scheme from dark green to dark red. CFS = Cell free supernatant, nCFS = neutral Cell free supernatant. Data presented are Mean ± SD of duplicated values obtained from independent trials.

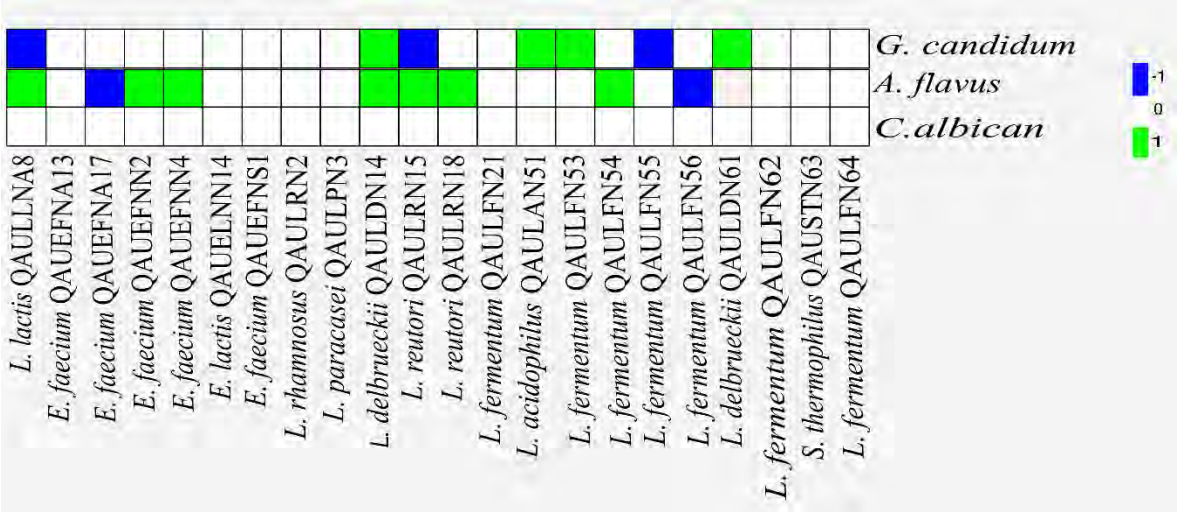

Figure 3. 11: The anti-fungal activity of probiotic bacteria isolated from Dahi samples against *G. candidum* QAUGC01, *A. flavus* and *C. albican* ATCC 90028. *Green color presents strong activity; blue color presents moderate activity while white color presents no activity.

3.3.2.5 Antibiotic susceptibility

The isolates were checked for their susceptibility toward various antibiotics purchased from Sigma Aldrich. For a suitable functional probiotic, two sets of antibiotics are usually suggested through EFSA rules like cell wall inhibitors and protein synthesis inhibitors. The results were related to the diameter of zones inter-operative chart given in the catalog. Inconstant antibiotic sensitivity was detected in all the isolates and are stated consequently in Figure 3.12.

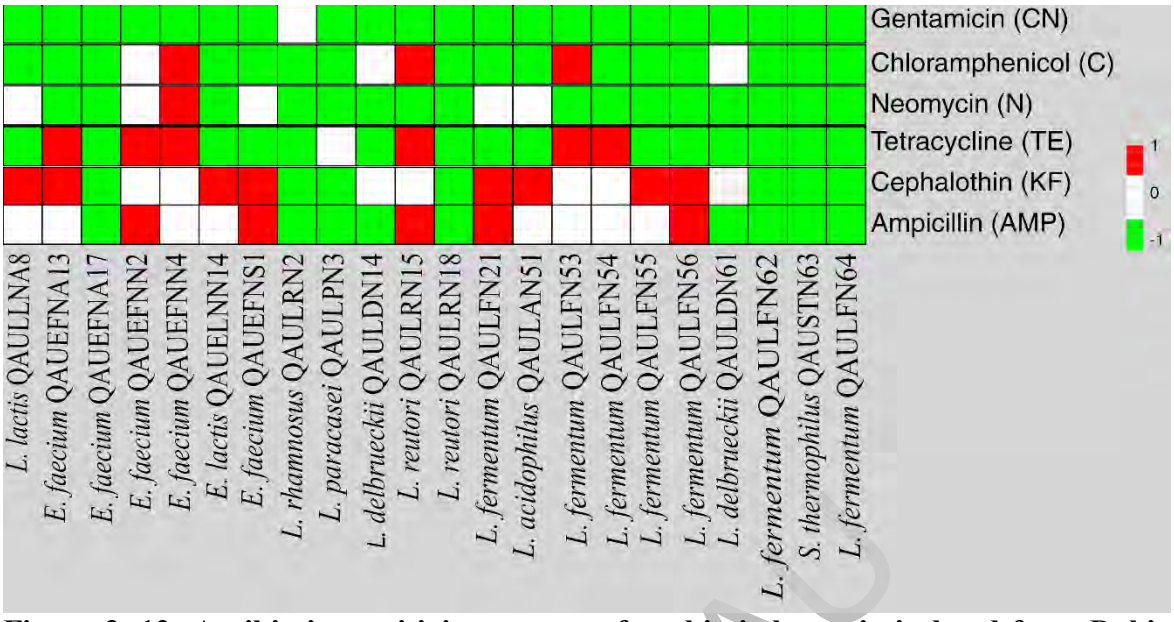

Figure 3. 12: Antibiotic sensitivity pattern of probiotic bacteria isolated from Dahi samples. Green color presents Susceptible; White color presents Intermediate while red color presents Resistant. The breakpoints for Antibiotic sensitive/resistant in mm zone of inhibition: AMP (≥17/≤14), C (≥18/≤12), KF (≥18/≤14) TE (≥19/≤14), CN (≥15/≤12), and N (≥17/≤12)

3.3.2.6 Bile Salt Hydrolase activity

Bile salt hydrolase activity (BSH) of the stains helps in determining their role in lipid metabolism and for the generation of short-chain fatty acids. The strains with potential probiotic properties were evaluated for their BSH activity. Some of the strains showed BSH activity, while some did not present any activity. Detail results are presented in Figure 3.14.

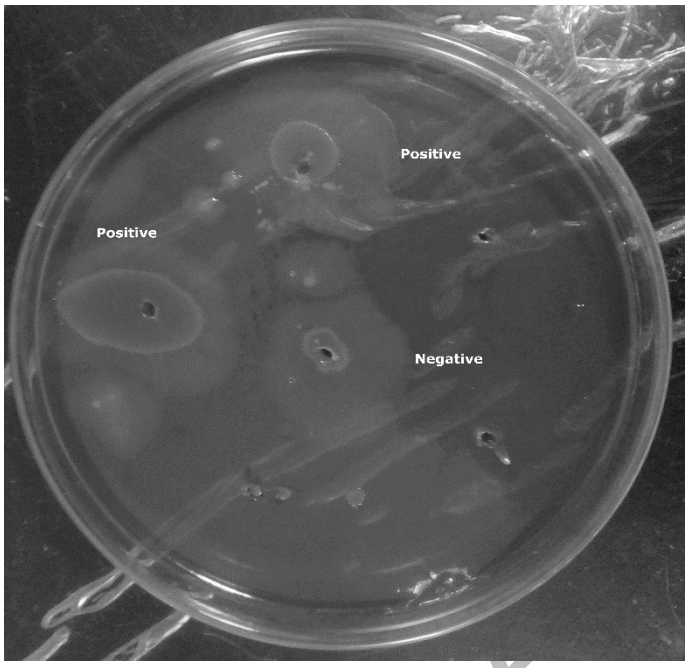

Figure 3. 13: Bile salt hydrolase activity of probiotic bacteria isolated from Dahi samples. Precipitation around the colonies present positive results while colonies with no precipitation presents negative results.

3.3.2.7 The *in vitro* cholesterol assimilation

The ability of potential probiotic isolates to assimilate cholesterol in the *in vitro* environment could help to be used for the management of body weight, obesity, and related diseases. The bacterial isolates were evaluated for *in vitro* cholesterol assimilating ability. All the isolates had excellent cholesterol assimilating ability ranging from a minimum of 84% for *L. rhamnosus* QAULRN2 to a maximum of 100% for *E. faecium* QAUEFNA13, *E. faecium* QAUEFNA17, *E. faecium* QAUEFNN14, and *E. lactis* QAUELNN14.

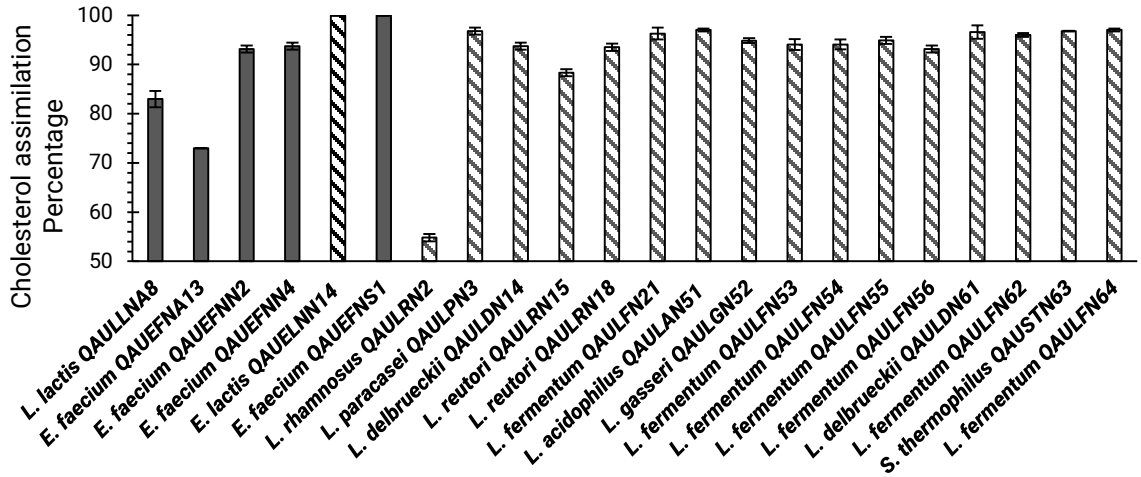

Figure 3. 14: The *in vitro* cholesterol assimilating potential and bile salt hydrolase activity of probiotic bacteria isolated from Dahi samples after 24 hours of incubation at 37°C. Black filed bars present negative hydrolase activity; pattern filled bars presents positive hydrolase activity. Percentage cholesterol assimilation is presented on scale. Data presented are Mean ± SD of duplicated values obtained from independent trials.

3.3.2.8 Antioxidant activity of the isolates

All the isolates were checked for Hydroxyl radical scavenging ability; all the isolates presented different patterns. The isolate *L. fermentum* QAULFN21 presented maximum ability of 76%, while the isolate *L. lactis* QAULLNA8 presented minimum ability of 37%. The antioxidant ability of potential probiotic isolates is presented in Figure 3.15.

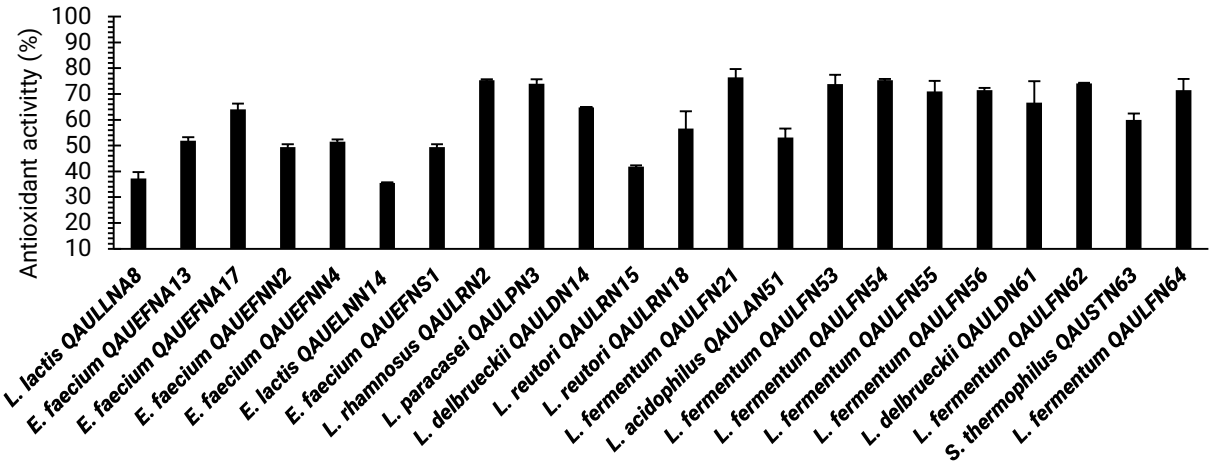

Figure 3. 15: The *in vitro* antioxidant activity of probiotic bacteria isolated from Dahi samples. Data presented are Mean ± SD of duplicated values obtained from independent trials.

3.3.3 Safety evaluation of the isolates

Safety of the isolates was determined by evaluating their hemolytic, DNase, and Decarboxylase activities as shown in Figure 3.16. The results revealed no hemolytic, no DNase, and no Decarboxylase activity of the isolates, that further confirms the non-pathogenic and non-toxicogenic nature of the selected LAB isolates.

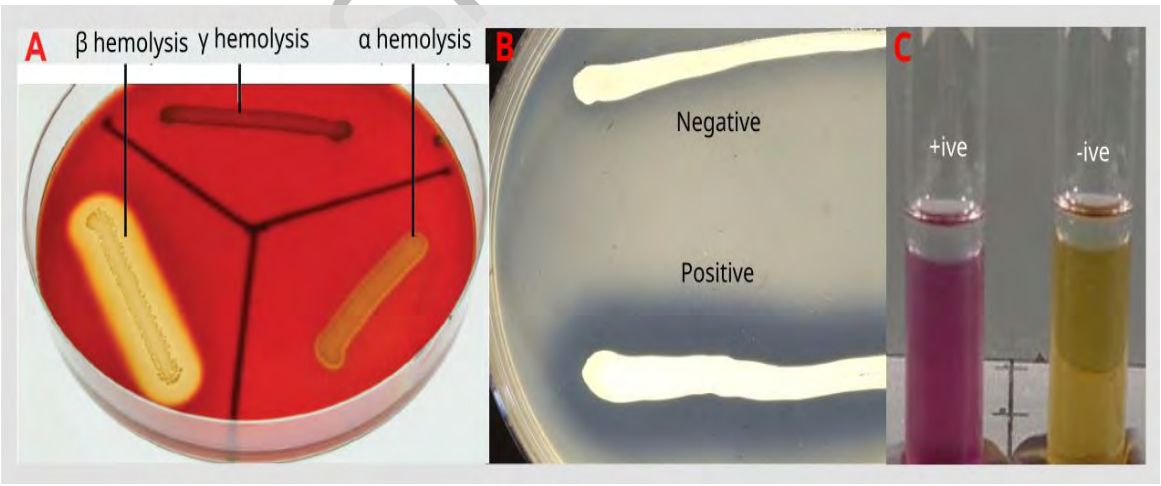

Figure 3. 16: Safety evaluation of the bacterial isolates (A) Hemolytic activity (B) DNase activity (C) Decarboxylase activity

3.3.4 Technological characterization of the isolates.

3.3.4.1 Enzymatic potential of the isolates

All the isolates were tested for production of different dietary enzymes by inoculating in specified media for 24 hours. The enzymes production profile of all the isolates is presented in Figure 3.17. All the strains presented a varied pattern for enzymes production. Among the 22 tested strains 13 were proteolytic, 10 where amylolytic, 5 were lipolytic and 15 were cellulolytic.

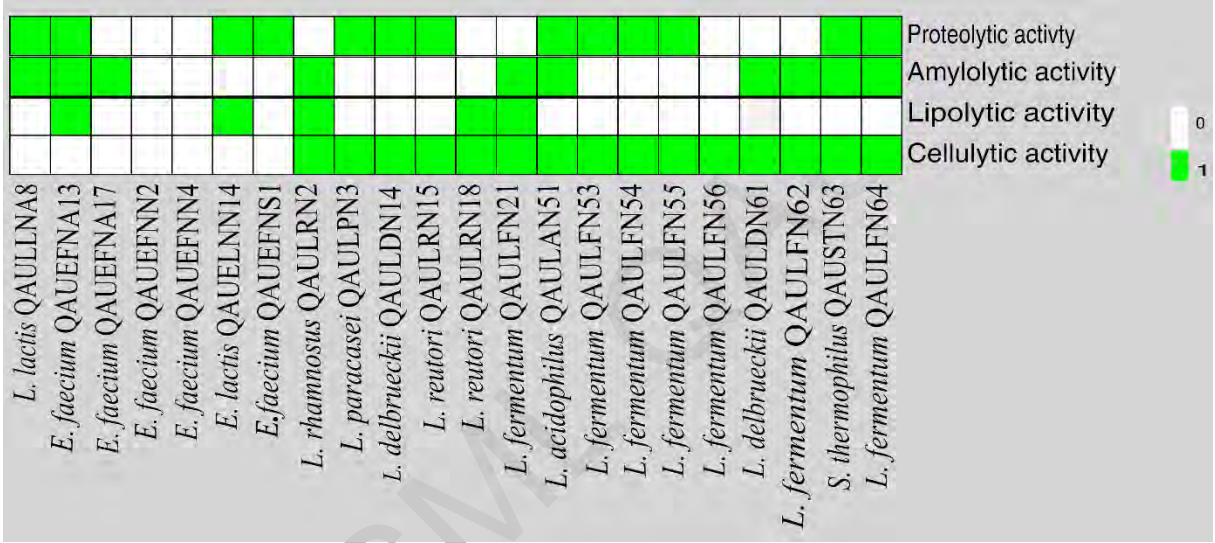

Figure 3. 17: Enzymatic potential of probiotic bacteria isolated from Dahi samples. The figure is presented in color scheme, white color presents negative results while green color presents positive results.

3.3.4.2 Survival of the isolates at different salt concentrations

All the isolates were checked for their ability to tolerate different salt concentrations like 4 and 6% for *Lactobacillus* species and 2 and 4% for *coccus* species. The strains were able to tolerate mentioned concentration of salts. Results are presented in Figure 3.18 - 3.21 respectively.

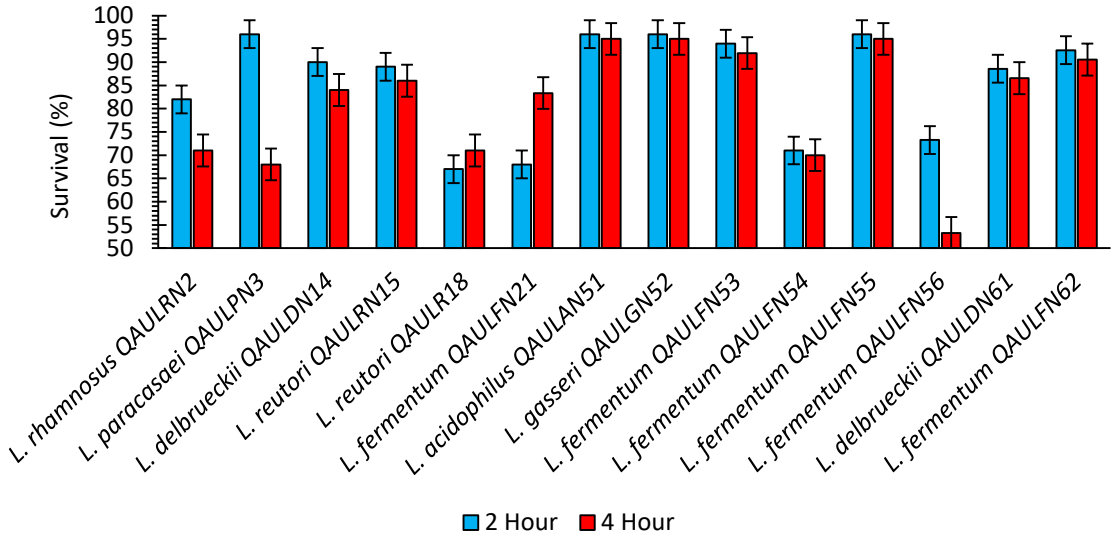

Figure 3. 18: Percentage survival of probiotic bacteria isolated from Dahi samples in 4% NaCl after exposure of 2 and 4 hours at 37°C. Data presented are mean ± SD of duplicated values obtained from independent trails.

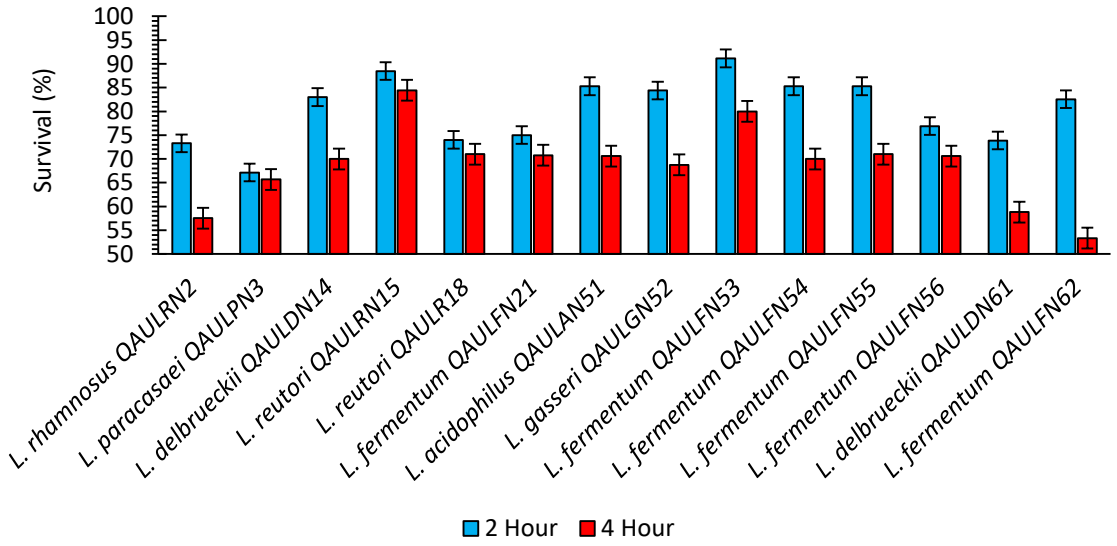

Figure 3. 19: Percentage survival of probiotic bacteria isolated from Dahi samples in 6% NaCl after exposure of 2 and 4 hours at 37°C. Data presented are mean ± SD of duplicated values obtained from independent trails.

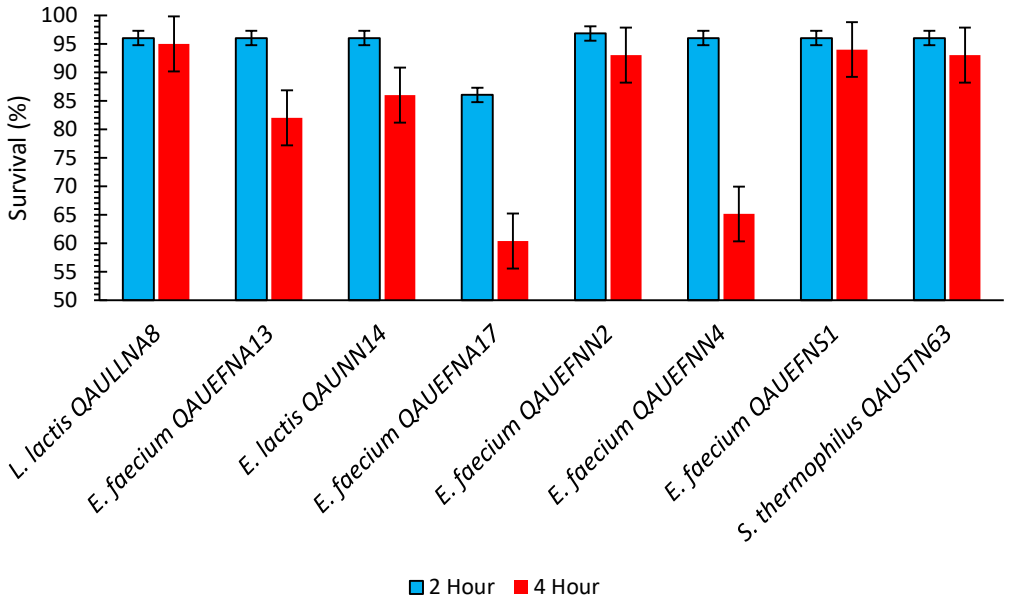

Figure 3. 20: Percentage survival of probiotic bacteria isolated from Dahi samples in 2% NaCl after exposure of 2 and 4 hours at 37ºC. Data presented are mean ± SD of duplicated values obtained from independent trails.

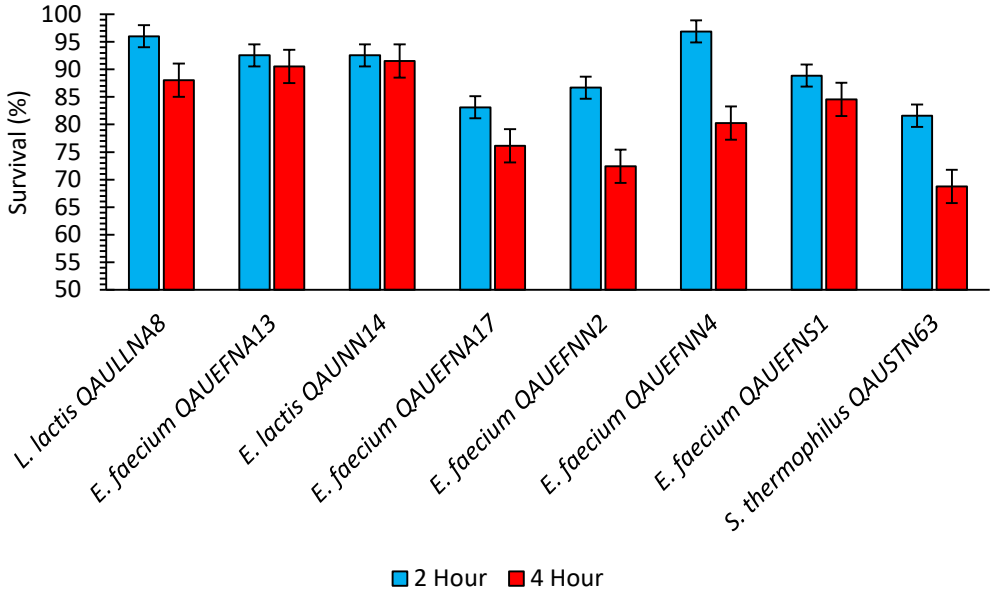

Figure 3. 21: Percentage survival of probiotic bacteria isolated from Dahi samples in 4% NaCl after exposure of 2 and 4 hours at 37ºC. Data presented are mean ± SD of duplicated values obtained from independent trails.

3.3.4.3 Growth of the isolates at different temperatures

All the isolates were checked for their ability to grow at both low (15ºC) and high (45ºC) temperatures. Though their growth pattern was different, all the isolates were able to grow in the respective temperatures. The growth pattern of all the strains is presented in Figure 3.22.

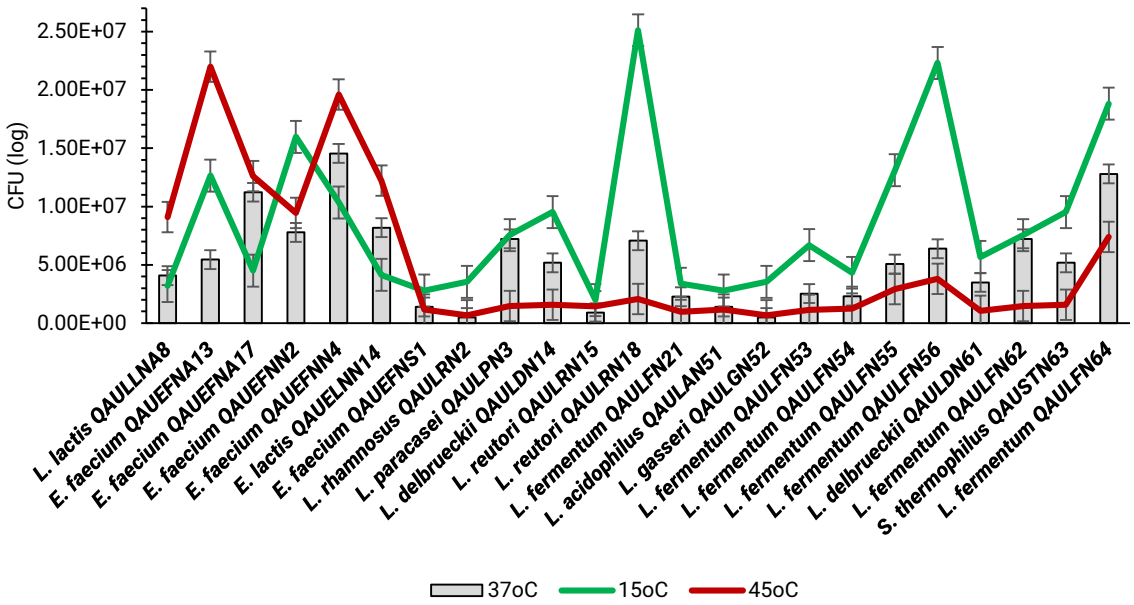

Figure 3. 22: Growth of probiotic bacteria isolated from Dahi samples at 15ºC, and 45ºC after incubation of 24 hours in comparison to 37ºC. Data presented are Mean ± SD of duplicated values obtained from independent trials.

3.4 Discussion

The traditional fermented milk product Dahi is having unique typicity related to sensory and therapeutic attributes, is the oldest and most consumed dairy product in Pakistan and neighboring countries (Masud *et al.*, 1991a; Younus *et al.*, 2002). It has been widely accepted in the local community that Dahi has therapeutic characteristics. The safety, therapeutic and sensory attributes of Dahi can be linked to the diverse microbial communities present in the Dahi (Burke *et al.*, 2018; Hummel *et al.*, 2007; Oliver *et al.*, 2005). Bacteria strains present in the Dahi, if isolated and characterized, can be used as probiotics for therapeutic purposes and in the formulation of functional foods. Aiming this,

the present study was designed to isolate, identify, and characterize potential native probiotic bacteria from artisanal fermented Dahi.

Initially, 143 bacterial isolates were selected for purification, identification, and characterization using specific media. Fifty-five isolates were Gram-Negative while 88 were Gram-positive. Eighteen Gram positive isolates were found catalase positive while seventy isolates were Gram-positive cocci or bacilli, non-motile, catalase, and oxidase negative. These types of primary characterizations have also been reported for the bacterial isolates of artisanal fermented milk products (Bhattarai *et al.*, 2016; Maqsood *et al.*, 2013; Nawaz *et al.*, 2016). Lactic acid bacteria, the dominant genera of fermented products are known prominently for acid production (Bozoudi *et al.*, 2015). When evaluated for acid production ability, it was observed that all the seventy isolates produced acids in the presence of $CaCO_3$ in the agar media. The isolates were also tested for lactose utilization by providing lactose as the only carbon source in culture media with phenol red (acid-base indicator). It was observed that all the isolates utilized lactose. These isolates were further tested for curd forming abilities via inoculating them in pasteurized milk for 6 – 8 hours at 42ºC. It was observed that 62 out of 70 isolates were able to curd the milk. These properties justify the presence of lactic acid bacteria in the milk as it contains 4-6% of lactose that is utilized by lactic bacteria as a carbon source, converting it to a final product lactic acid (Bozoudi *et al.*, 2015; Mora *et al.*, 2018; Pereira & Paula, 2014; Widyastuti & Febrisiantosa, 2014). Production of acids, utilization of lactose, and curdling of milk givens phenotypic confirmation of the isolates as lactic acid bacteria. When Rep-Sep technique which identifies bacteria even at strain level (Krych *et al.*, 2019), was used for molecular identification of the bacterial isolates it was found that 62/70 (88%) isolates belong to the lactic acid bacteria. The *L. fermentum* was the most abundant 23/62 (37%) of the isolated bacteria. These results are supported by a study where *L. fermentum* was abundantly isolated from a traditional fermented milk product of Kenya (Mathara *et al.*, 2004). The *E. faecium* was second most abundant (17/62) bacteria isolated from Dahi. These finding are also supported by a study where *E. faecium* is abundantly isolated from traditional fermented milk products Dahi and Datshi of Bhutan (Shangpliang *et al.*, 2017). Other

isolated lactic acid bacteria confirmed through Rep-Seq were as *E. lactis* (n = 2), *L. lactis* (n = 3), *S. thermophilus* (n = 2), *L. acidophilus* (n = 2), *L. plantarum* (n = 2), *L. paracasae* (n = 2), *L. casae* (n = 2), *L. gasseri* (n = 2), *L. delbrueckii* (n = 2) and *L. reutori* (n = 2), these findings are in line with some recent studies on the isolation and identification of lactic acid bacteria from traditional fermented milk products (Mathara *et al.*, 2004; Shangpliang *et al.*, 2017). In current study, the presence of *B. subtilis* (n =1), *B. mycoides* (n =3) and *B. licheniformis* (n = 4) were also confirmed through Rep-Seq method. These findings are also supported by a recent study where different species belonging to genera *Bacillus* are isolated along with lactic acid bacteria from different types of fermented foods (Cissé *et al.*, 2019).

Resistance to bile salts, fluctuating pH, and different temperatures are the prerequisite for potential probiotics for their survival in the host's intestine and food's environment (Ho & Sze, 2018; Jatmiko *et al.*, 2017). For this purpose, the isolates were exposed to low pH i.e. (2 and 5) where 56/70 (80%) isolates were able to grow at low pH of 2. These findings are in line with recent findings where lactic acid bacteria isolated from dairy ecosystem were able to grow at pH 2 (Eshaque, 2018). When exposed to bile salts, 49 isolates (70%) showed more than 50% survival rate in 0.45% bile salt supplemented media in comparison to no bile salt media. Studies reported that probiotic bacteria isolated from dairy and cheeses are resistant to 0.3% of bile salts (Hassanzadazar *et al.*, 2012; Zoumpopoulou *et al.*, 2018). Similar results were also reported earlier for the strains isolated from milk and yogurts (Ramana & Pravin, 2015). Cell surface hydrophobicity (SCH) of microorganisms plays an important role in attaching to or detaching to or from the surfaces. Hydrophobic bacteria have more potential for attachment to surfaces than hydrophilic bacteria. For attachment to the intestinal lining, lactic acid bacteria must have a high rate of *in vitro* hydrophobicity (Krasowska & Sigler, 2014; Puniya *et al.*, 2016). The *in vitro* cell surface hydrophobicity for all the bacteria isolates was evaluated. Moreover, to protect themselves from environmental stress agents or host responses, bacteria form clumps as a defensive strategy, hence clumps forming ability of all the isolates through *in vitro* auto-aggregation test was evaluated. The evaluated isolates (n = 49) had *in vitro* cell surface hydrophobicity

and auto-aggregation abilities above 50%. Our findings are supported by recent studies, where low, as well as high hydrophobicity and auto-aggregation for individual strains of lactic acid bacteria, have been reported (Angmo *et al.*, 2016; Guo *et al.*, 2010; Kathiriya *et al.*, 2018).

Survival of the isolates in the *in vitro* simulated intestinal fluids was evaluated via incubating in the simulated fluids for 4 hours at 37ºC and 120 RPM. All the isolates (n = 49) were able to survive in simulated salivary fluid but only 22 isolates were able to survive in the simulated gastric fluids. It has been found that lactic acid bacteria isolated from the dairy environment are able to tolerate the gastric environment (Campana *et al.*, 2017).

Lactic acid bacteria produce lactic acid, thus lowering the media's pH and producing antipathogenic compounds or peptides acting antagonistic to pathogenic microorganisms (Pisoschi *et al.*, 2018). Antagonistic activity of the isolates against known pathogenic ATCC strains of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Streptococcus pneumoniae,* and *Bacillus subtilis* was evaluated. All the isolates showed antagonistic activity against the aforementioned pathogens at a various rates ranging from 51% to 88%. Similar results have been reported that lactic acid bacteria isolated from dairy products have antagonistic activity against food-borne pathogens *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli* and *Salmonella enterica* (Leite *et al.*, 2015; Tsai *et al.*, 2008).

A factor of safety probiotic must be sensitive to daily used antibiotics that have been recommended by EFSA (Álvarez-Cisneros & Ponce-Alquicira, 2018). The isolated bacterial isolates were tested for susceptibility toward the recommended antibiotics like Gentamicin, Chloramphenicol, Neomycin, Tetracycline, Cephalothin, and Ampicillin. All the isolates presented a different susceptibility pattern toward the tested antibiotics, but no isolate was observed as multi drug or extreme drug resistant. It has been reported that lactic acid bacteria isolated from fermented dairy products are sensitive to different antibiotics (Lee *et al.*, 2009; Ren *et al.*, 2018; Soltani *et al.*, 2016). Lactic acid bacteria along with other compounds can produce biogenic amines which are responsible for many adverse effects and pathological syndromes in humans (Barbieri *et al.*, 2019; Pessione &

Cirrincione, 2016). Among various known biogenic amines, arginine, histamine and tyrosine are the most dangerous amines in pathogenicity and toxigenicity (Hungerford, 2010; Mccabe-Sellers *et al.*, 2006). These amines are associated with dairy and microorganisms of dairy origin (Benkerroum, 2016). For safety assessment bacterial isolates were checked for biogenic amines production especially arginine and tyrosine. It was seen that all the isolates were negative for arginine and tyrosine production. Studies also reported biogenic amines producing lactic acid bacteria isolated from dairy and fermented products (Jin *et al.*, 2019; Ladero *et al.*, 2015; Pessione & Cirrincione, 2016). Probiotics can be helpful when consumed alive and taken alive when proven to be safe. For this purpose, the isolated strains were checked that how to react to the blood if provided in the media. It was observed that all the strains have no homolysis activity. Recently, a study reported that lactic acid bacteria isolated from dairy products have no homolysis activity on blood agar (Zarour *et al.*, 2018). To ensure that the isolates are non-pathogenic in nature, these were tested for DNase ability which is specifically associated with pathogenic bacteria. It was observed that all the isolates have no DNase activity. The same results have been recently published where the safety of probiotics isolated from dairy products has been evaluated through DNase activity (Zarour *et al.*, 2018).

Proteolytic, lipolytic, amylolytic, and cellulolytic activities of all the isolates were tested. Thirteen isolates out of twenty-two had proteolytic activity on milk agar plates. It has been documented that lactic acid bacteria have complex proteinase and peptidase systems and use milk proteins as a source for essential and growth-promoting amino acids. Lactic acid bacteria have ability to break 40% of α_{S1}-CN and β-CN bonds to release more than a hundred oligopeptides from milk proteins (Choi *et al.*, 2012). However, extracellular proteolytic activity has been reported for *Lactococcus bulgaricus* and *Lactococcus lactis* (Addi & Guessas, 2015; Lim *et al.*, 2019). These peptides act as energy sources for other synergistic bacteria, precursors for flavoring compounds, or as antibacterial peptides for pathogenic and undesirable microorganisms. Our findings agree with previous studies that reported proteolytic activity of *Streptococcus, Lactococcus, Enterococcus,* and *Lactobacillus spp* isolated from dairy ecosystems (Abushelaibi *et al.*, 2017; Mora *et al.*,

2018; Pontonio *et al.*, 2019). Five isolates belonging to *E. faecium, E. lactis, L. rhamnosus, L. reutori* and *L. fermentum* showed lipolytic activity, while the remaining isolates were non-lipolytic, when tween 80 was used as a substrate in the media. These finding were similar to the reports of lipolytic *E. faecium, E. lactis,* and *lactobacillus* isolated from milk and cheeses, but the number of non-lipolytic lactic acid bacteria was more significant to the number of lipolytic bacteria (Blaya *et al.*, 2018; Formisano *et al.*, 1974; Hanchi *et al.*, 2018a; Katz *et al.*, 2002; Murugan & Villi, 2009; Simonová *et al.*, 2008). It has been documented that genetically lactic acid bacteria have low potentials for fats and lipids degradation (Dinçer & Kıvanç, 2018; Mcauliffe, 2017). Different methods for screening of lipolytic bacteria have been reported, and plate assay is considered the most rapid and economical method. However, screening methods could lead to differences in the results (Daroonpunt *et al.*, 2018; Samad *et al.*, 1989). Strains with high lipolytic activity can be used as starter or adjunct cultures and can also be used for cholesterol assimilation activities in the *in-vivo* (Dora & Glenn, 2002). Strains belonging to the species of *S. thermophilus*, *L. lactis, E. lactis, E. faecium,* and *Lactobacillus* were amylolytic positive. It has been reported that the amylolytic lactic acid bacteria are able to degrade the starch and convert it into low molecular weight sugars that have many industrial applications (Giraud *et al.*, 1994; Mehta & Satyanarayana, 2016). Amylolytic lactic acid bacteria are naturally present in fermented foods, plants matrixes, plant wastes, and the digestive tracts of humans and animals (Ray & Montet, 2016). Studies have been reported for isolating amylolytic *S. thermophilus, E. faecium* and *E. lactis* from dairy products (Díaz-Ruiz *et al.*, 2003; Harun-Ur-Rashid, Togo*, et al.*, 2007a; Marcela *et al.*, 2016; Oglu Gulahmadov *et al.*, 2006). It has been stated that milk naturally has no cellulolytic bacteria (González *et al.*, 2010; Ho & Yin Sze, 2018). While rumen of ruminants is a natural habitat for cellulolytic bacteria (Adjei-Fremah *et al.*, 2018; Foong *et al.*, 1997). 15/22 isolates in the present study were observed as cellulolytic. The cellulolytic lactic acid bacteria may have been introduced to the milk during milking or processing of the milk (Haghshenas *et al.*, 2017).

Growth at various temperatures is also used as a method for lactic acid bacterial classification and characterization and is very basic to applications in industry (Hammes

& Vogel, 1995). The *Lactobacilli* are unable to grow at 15°C, but they can grow at 45°C. In the current study, we tested the growth of all bacterial isolates at 15 and 45ºC temperatures. When compared to the growth at ambient temperature, it was observed that all the isolates showed growth at 15ºC and 45ºC. However, *Lactococcus* isolates, and *Enterococcus* showed low growth at 45ºC. Current findings align with the early reports (Daeschel & Fleming, 1984; Papadimitriou *et al.,* 2016; Zhou *et al.*, 2016). The isolates were also evaluated for tolerance to 2, 4 and 6% NaCl. All the tested isolates (22) were able to tolerate the tested concentration of NaCl.

3.5 Conclusion

Microbiology of the traditional fermented milk product Dahi is diverse that contain lactic acid bacteria as the prominent group. Lactose utilization test, acid production test, and curd formation can be used in the initial biochemical screening for LAB. The isolates which were positive for traits were LAB when confirmed through Rep-Seq identification technique. The LAB isolates that were able to survive in low pH, high bile salts and presented high *in vitro* CSH and *in vitro* AAG were not able to tolerate the *in vitro* simulated gastric fluids. The antagonistic activity of lactic acid bacteria toward different pathogens is strain dependent. The *in vitro* safety assessment confirms the GRAS status of LAB isolated from fermented milk product Dahi. Survival in low and high pH and resistance to different salts concentrations enables the probiotic LAB for use in food items. However, this Phase (Phase I) of the study only concerns the genetic analysis and phenotypic characterization of individual bacterial species found the Dahi samples.

Chapter 4
Selection, Functional and Genomic Characterization of Multi-Strains Probiotic Community

4.1 Introduction

Probiotics are defined as viable microbes that, when administered in adequate amounts, confer health benefits on the host" (Sanders, 2008). For desired health benefits, probiotics are given to the target host either in supplements containing only live, dead, or some parts of probiotic microorganisms, functional foods mainly containing 1 to 2 specific strains or in the form of biologically fermented dietary products (Markowiak & Śliżewska, 2017). Dairy products that are industrially fermented and formulated often contain from 1 to 10 or slightly more strains of some well-characterized probiotic candidates. However, in contrast, dairy products traditionally fermented harbour a mixture of up to hundreds of unidentified and uncharacterized microorganisms. Although there is no modern way to mimic traditional fermentation, still their health benefits are broad, adverse effects are less, and consumer preferences are comparatively high due to unique taste and flavor (Macori & Cotter, 2018). Hence, it is why the diary supplementation market is inclined toward the formulation of products with more probiotic strains. The multi-strain formulation has different strains in different proportions. But the overall viable count of multi-strain probiotics has been considered a common practice for quality satisfaction (García-Burgos *et al.*, 2020). Several possible benefits have been believed to be linked with the use of multi-strains probiotics, as compared with single-strain probiotics. Such as multi strains might have maximum chances of beneficial success and hence have a greater success rate for the desired health benefit. Multi strains offer a high diversity and thus have more possible functions and a broader range of effectiveness (Ouwehand *et al.*, 2018). Multi-strain probiotics should have preservative or synergistic effects: greater and strong attachment, formation of a favorable living environment, and declined antagonism of endogenous microbiota (Kwoji *et al.*, 2021). For example, a multi-strains probiotic consortium from cheese origin presented significant reduction of *Listeria monocytogenes* where the individual strains had no anti *listeria* activity (Imran *et al.*, 2013; Imran *et al.*, 2010).

In order to introduce new probiotics into the food or feed, these must be evaluated for safety both at phenotypic and genotypic levels (Doron & Snydman, 2015). The human

microbiota, specifically the intestinal microbiota, has been an "essential metabolic and functional organ," carrying more than 1000 bacterial species that collectively contain 150 times more genes than those coded by the complete human genome. Significant advances have revealed that the balanced intestinal microbiota is involved in essential human biological functions, including metabolism, controlling epithelial development, and improving innate immunity (Jandhyala *et al.*, 2015). In contrast, many metabolic and chronic diseases such as obesity, inflammatory bowel disease (IBD), diabetes mellitus, atherosclerosis, alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), cirrhosis, and hepatocellular carcinoma have been associated with dysbiosis and aberrant intestinal microbiota (Degruttola *et al.*, 2016). It has been proposed that dysbiosis gut microbiota-associated diseases can be treated through beneficial modulation of the gut microbiota. Many approaches like fecal material transplant, antibiotics, pre, and probiotics are used to modulate gut microbiota in some specific disease conditions. Probiotics offer an attractive or adjunct therapy for modulation of gut microbiota and treatment of various diseases. Number of studies have been reported a beneficial effect of single strain probiotics for various diseases like obesity, IBD, Diarrhea and many others (Satokari, 2019). It is also supposed that it might be difficult for a single strain to modulate a complex community of thousands of bacteria, and if used multi-strains, it might comparatively have more success rate (Hasan & Yang, 2019). However, the evidence that multi-strain probiotics are more effective than single strains is still limited.

In this context, the aim of current research was to develop, functionally and genomically characterized multi-strains probiotic community from individually characterized probiotics for future use.

4.2 Material and Methods

4.3 Selection and characterization of multi-strains probiotic community.

Based on two basic approaches such as diversity of probiotics and viability of probiotics in the simulated intestinal environment, two probiotic communities were chosen from Phase I of the study for functional and genomic characterization. One community consisted of all the strains that tolerated low pH, high bile salts, presented cell-surface hydrophobicity

and auto-aggregation. This community consisted of 49 isolates and was named as multi-strains probiotic community 1 **(MSPC-1)** (Strains listed in Figure 3.7 of Phase I), second community consisted of all those isolates that tolerated the simulated gastrointestinal fluids along with aforementioned factors. This community consisted of twenty-two LAB strains and was named as multi-strains probiotic community 2 **(MSPC-2)** (Strains listed in Figure 3.8 of Phase I)**.** To check which community behaves best, both the communities were subjected to growth determination, *in vitro* cell surface hydrophobicity, auto-aggregation, antibacterial activity, and *in vitro* cholesterol assimilating actives and the best community was chosen for further characterization.

4.3.1 Shared growth determination

Both MSPCs were evaluated for their synergistic interaction in terms of growth through conventional culturing techniques. For this purpose, two media were selected such as Tryptic soya mostly used general purpose medium for growth of lactic acid bacteria and skim milk medium. All the isolates were grown individually for 24 hours. Then 100 µl of each isolate comprising 10^8 cells were combined in a sterile tube. From this tube, 100 µl bacterial culture was taken and spreaded on TSA and SMA plates. These plates were incubated at 37ºC for 24 hours both aerobically and anaerobically. After 24 hours of incubation colonies on each plate were counted through digital colony counter and the results were expressed as CFU.

4.3.2 Cell surface hydrophobicity

The cell surface hydrophobic activity of MSPCs were assessed *in vitro* by measurement the microbe's grip to hydrocarbons through the assay explained by (Dlamini *et al.*, 2019). The fresh broth cultures were centrifuged for 10 mints at 8,000 × g and 4ºC, cleansed two times with Normal saline, and re-drooped in standard saline buffer, subsequently measured its absorbance (A0) at 600 nm. A 3 ml volume of bacterial cells was combined with 1 ml xylene and kept static at 37ºC for 1 hour, resulting in the appearance of two phases organic and aqueous. The aqueous part was taken off cautiously and recorded its absorbance (A1)

at 600 nm. The hydrophobicity percentage was recorded by a reduction in absorbance and measured with the equation:

$$CSF\ (\%) = 1 - \frac{A1}{A0} \times 100$$

4.3.3 Auto-aggregation

The capability of the MSPCs to auto-aggregate was measured as per (Dlamini *et al.*, 2019). Fresh culture was centrifugated for 10 min at 8,000 × g and 4ºC for 10 min, twice cleansed with saline, and re-suspended in saline. The sample was left to stand for a moment, incubating at 37ºC, and the aqueous layer was taken and measured for absorbance at 600 nm after 1 hour. The percent auto-aggregation was calculated with the formula.

$$AAG\ (\%) = 1 - \frac{AT}{A0} \times 100$$

Where AT shows the absorbance at a time, and A0 shows the absorbance at time 0.

4.3.4 Evaluation of antibacterial properties

The antibacterial effect of MSPCs were assessed through optical density measurement against *Bacillus subtilis* subsp. subtilis ATCC 19659, *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. enterica ATCC 27870, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. aureus ATCC 6538 and *Streptococcus pneumoniae* ATCC 49619. The fresh overnight culture of MSPCs were initially centrifugated (5000 × g,10 min, 4ºC), the supernatant was filtered with 0.2 µm syringe filter and divided into two equal parts. One part was used as it was extracted through centrifugation i.e., the cell-free supernatant (CFS). While for the second part, to remove the assumed outcome of organic acids present in the filtrate, the pH of the filtrate was adjusted to 6.5 using NaOH/HCl (n-CFS), and both the parts were stored at -20ºC for further scrutiny.

Then a sterilized 96-well plate was poured with 50 µl of pre-prepared filtrate, 50 µl of the respective pathogen, and 200 µl of Luria Broth to uphold a final concentration of 10^8 cells/well. The LB broth inoculated with pathogenic suspension was used as a positive control, and the LB broth deprived of any inoculation was used as a negative control. The well-plate was incubated at 37ºC for 24 hours, and the optical density (OD) was taken at 600 nm. The total percent pathogen inhibition was determined with the following equation:

$Antibacterial\ effect\ (\%) = \frac{OD\ contol - OD\ test\ sample}{OD\ control} \times 100$ (Georgieva *et al.*, 2015).

4.3.5 The *in vitro* cholesterol assimilating activity of MSPCs

To assess the *in vitro* cholesterol assimilation ability of MSPCs, the method described by (Madeeha *et al.*, 2016) was used with minor modification. A stock solution of the cholesterol and its dilutions were prepared. Solutions of 0.1 mg/ml to 5 mg/ml chloroform/cholesterol concentrations were prepared. From these, 0.1 ml was taken and was added to 9.9 ml of the $FeCl_3$ – acetic acid solution. Then were vortex and left for 15 minutes. Five ml of this solution was taken, and 3 ml of H_2SO_4 was added to it and vortex for 30 sec. the tubes were left for 30 minutes, and OD was taken at 560 nm and from this standard curve was made for known concentrations.

The MSPCs were grown in respective broth media for 15 hours, and 100 µl of the bacterial suspension was then transferred to 9.9 ml of fresh broth. The 0.4 mg /ml (0.1ml) of this solution was added into the test tube to make available 0.04 mg/ml to the microbial communities and were incubated at 37ºC for 24 hours. After incubation, 0.1 ml sample was taken from each test tube and added to the test tube having 9.9 ml of $FeCl_3$-acetic acid solution. The tubes were vortexed for 15 min, subsequently centrifuged at 8000 × g for 10 min. After centrifugation, 5 ml of the supernatant was taken in a separate sterile test tube, and 3 ml of H_2SO_4 was added into it and vortexed for 30 s. OD of the sample was measured at 560 nm after 30 min of incubation in the dark.

The cholesterol assimilation was measured with the following formula.

$$Cholesterol\ assimilate = (Initial\ cholesterol\ conc - Final\ cholesterol\ conc)$$

$$Cholesterol\ assimilated\ (\%) = \frac{Cholesterol\ assimilated}{Total\ cholesterol\ conc\ in\ media} \times 100$$

4.3.6 Survival of MSPC in TSI *in vitro* intestinal model

The survival of MSPC in the *invitro* simulated small intestine was evaluated by inoculating the MSPC in the model and then through plate count and metagenomics profile. The overview of the model and sampling points are presented in Figure 4.1.

As described by (Cieplak *et al.*, 2018), the small intestine (TSI) is a small *in vitro* model mimicking the stomach and small intestine. The TSI model is basically composed of five reactor vessels with a working volume of 12 ml in each reactor. Bile is absorbed during the simulated passage through the SI, and pH is adjusted to physiologically relevant values for the duodenum, jejunum, and ileum. The model also contains a microbial consortium native to the ileum.

4.3.6.1 Running the simulation

Before the TSI run, freshly prepared simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were preheated by putting in 50 ml ITS tubes in TSI at 37ºC overnight. The gastric and intestinal media were adjusted to pH 2.5 and 6.5, respectively. The reactor chamber was assembled, and 1.1 ml of feed/water (for fed or fast phase, respectively), 1.4 ml of SSF, 2.3 ml of SGF, and 1.4 µl of 0.3M $CaCl_2$ were added. An anaerobic sachet was put in the reactor, and the lid was closed tightly, the pH probes were blocked with plastic sticks and placed inside TSI, the magnetic stirring was adjusted at 250 rpm. A dialysis chamber was prepared by adding autoclaved Milli-Q® water into the chambers, and a dialysis cassette was connected to it and the lids. To promote anaerobic conditions, 2 g/L cysteine was added to the water, and to maintain anaerobic conditions anaerobic sachet was placed inside the smaller compartment of the camber.

The temperature was adjusted to 37ºC, and for maintaining the temperature, the connected water bath was enabled for remote sensing. Computer-controlled pH meter and 0.5 M NaOH dosing syringe pumps were connected to the reactor vessel for pH regulation.

4.3.6.2 Stomach phase

For the stomach phase, 0.5ml of 2000 U/ml pepsin solution and 0.5 ml of probiotic community were added to the reactor through sterilized syringe and the simulation was allowed to run for 30 min in the fasting and 60 min for feeding phase.

4.3.6.3 Duodenum phase

At this step, for fasting phase, 0.32 ml of bile salts, 0.6 ml of pancreatic juice, and 10 µl of $CaCl_2$ were mixed with 4.7 ml of SIF while for feeding phase 0.8 ml of bile salts, 1.5 ml

of pancreatic juice, and 10 µl of $CaCl_2$ was added to 3.3 ml of SIF. The pH of this mixture was adjusted to 6.

4.3.6.4 Jejunum phase

At step, dialysis was started and allowed to run for 4 hours.

4.3.6.5 Ileum phase

At this step, 4 ml concentrated intestinal media and 1 ml of defined ileum bacterial consortia (Cieplak *et al.*, 2018) (list is also provided in Appendix) were added to the reactor vessel and allowed the simulation to run for 2 hours.

4.3.6.6 Sampling

At each stage, 200 µl of the sample was taken in a sterilized microtube and used for further analysis.

4.3.6.7 Determination of survival through culturing method

The 100 µl of the fermentation liquid was spread on Tryptic soy agar media plates and was incubated at 37ºC for 24 hours in aerobic and anaerobic conditions. After 24 hours of incubation, colonies present on each plate were counted through a digital colony counter and were expressed as CFU/ml

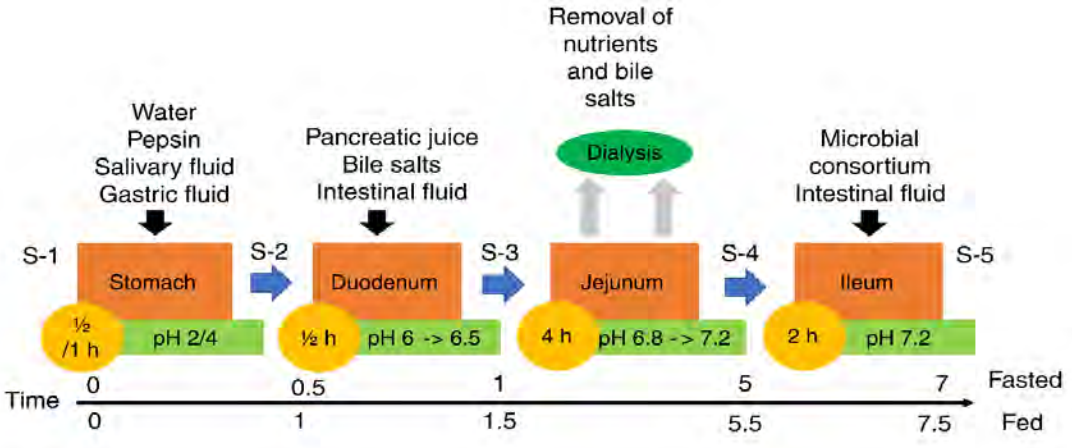

Figure 4. 1: Overview of the TSI model and detail of the sampling points (S-1 to S-5 are sampling points)

4.3.6.8 Evaluation of survival through culture independent method

4.3.6.8.1 DNA extraction

All the colonies present on each media agar plate were collected in a sterile 1.5 ml microtube, and the Bead-Beat Micro AX Gravity Kit (A&A Biotechnology, Gdynia, Poland) was used for the DNA extraction according to the manufacturer's protocol. Briefly, 450 µl of BS suspension buffer was added to the cells containing microtube. Subsequently, 20 µl of lysozyme and 5 µl of Mutanolysin were added. The sample was mixed well and incubated at 50ºC for 20 min. After 20 min of incubation, 900 µl of LSU lysis buffer and 20 µl proteinase K were added. Then the suspension was transferred into bead beat tubes having 1 g of Zicronica/Silica 1 mm beads; the sample was transferred into a bead-beater and run for 60 sec at maximum power. The mixture was then incubated for 30 minutes at 50ºC, vortexing from time to time. After the incubation, the sample was centrifuged at 12000 rpm for 5 minutes, and 1 ml of the supernatant was loaded onto the assembled and pre-equilibrated (by loading 500 µl of KIG solution) Micro AXD column and allowed to pass through the column through gravity. Then 600 µl of WIG first wash solution was added and allowed to pass through the column, and then the second wash solution W2 of 500 µl was added to the column. When the wash two solution was wholly passed through the column, 60 µl of E elution buffer was added onto the column and allowed to stand for 5 minutes. Finally, the extracted DNA was eluted into 1.5 ml sterilized microtubes having 5 µl of N neutralizing buffer with 120 µl of Elution buffer. The concentration and purity of the extracted DNA were measured using Nano-Drop ND-1000 spectrophotometer (Saveen and Werner AB, Sweden).

4.3.6.8.2 16S rDNA amplicon sequencing

MinION (Oxford Nanopore Technologies, Oxford, UK) was used to sequence the 16S rDNA. Prior to library preparation the extracted DNA was diluted to 10 ng/µl and the V1-V8 hypervariable region of 16S rRNA gene was amplified and sequenced with ONT using the following primers

ONT_27Fa: GTCTCGTGGG CTCGGAGATG TGTA TATAGA TCGCAGAGTT TGATYMTGGCTCAG, ONT_27Fb: GTCTCGTGGG CTCGGAGATG TGTA

TATAGA TCGCAGAGTT TGATCCTGGCTTAG and ONT_1540_R: GTCTCGTGGG CTCGGAGATG TGTA TACTCT CTATTACGGY TACCTTGTTACGACT.

4.3.6.8.3 PCR-1 ON_UMI16S

The purified extracted DNA was diluted with sterilized Milli-Q® water to 1ng/µl. PCR mix was prepared using PCRBIO Ultra Mix 12 µl, Sterilized Milli-Q® water 6 µl, Primer Mix 2 µl and 5 µl DNA for each sample. PCR was performed with 95ºC for 5 minutes. The 2 cycles of 95ºC for 20 seconds, 48ºC for 30 seconds, 65ºC for 10 seconds and 72ºC for 45 seconds followed by 72ºC for 4 minutes.

After completing PCR-1, the amplified DNA was visualized through 2% gel, and the quantity of DNA was measured through Quiblet. The PCR product was cleaned by transferring 18 µl of binding beads to 25 µl of PCR product and incubating at room temperature for 5 minutes. The product was washed twice with freshly prepared 80% ethanol.

4.3.6.8.4 PCR-2 ON_UMI16S

The 11 µl of the cleaned PCR-1 product was added to 12 µl of PCRBIO Ultra Mix and 2 µl of ONT barcodes (10 µM) into PCR tubes for second PCR. Moreover, PCR 2 was run at 95ºC for 2 min, 33 cycles of 95ºC for 20 s, 55ºC for 20 s, and 72ºC for 40 s, with the final step of 72ºC for 4 min. The DNA obtained from PCR 2 was visualized with 2% gel, and the quantity was measured with Quiblet. The PCR 2 product was pooled for library preparation and was cleaned with AMPure XP beads (Beckman Coulter Genomic, CA, USA). The pooled amplicons were exposed to 1D genomic DNA by ligation protocol (SQK-LSK109) to complete library preparation for MinION sequencing. Estimated 0.2 µg of amplicons were used for the initial step of end-prep. And 40 ng of prepared amplicon library was loaded on a R9.4.1 flow cell.

4.3.6.8.5 Sequencing data analysis

Data produced by MinION were assembled using Min-Know software v19.06.8 (https://nanoporetech.com). The Guppy v3.2.2 basecalling toolkit was utilized to base call raw fast5 to fastq (https://nanoporetech.com). Porechop v0.2.2 was used for adapter

trimming and sample demultiplexing (https://github.com/rrwick/Porechop). The Porechop adapter list was (adapters.py) edited accordingly. The quality scores of fastq files were corrected through NanoFilt (q ≥ 10; read length >1Kb). Taxonomy assortment of quality corrected reads compared to Greengenes (13.8) collection was performed by uclast method executed in parallel_assign_taxonomy_uclust.py (QIIME v1.9.1). The uclust settings were tuned on mock communities (−-similarity0.8; min_consensus_fraction 0.51) making sure annotations to the lowest possible taxonomic level with no false positive results. The settings permitted it to consider separate amplicon sequence options as separate "seeds" (Hui *et al.*, 2021). Reads categorized to at least phylum level was exposed for further analysis. The numbers of operational taxonomic units (OTUs); a collection of 16S rRNA sequences that have a certain percentage of sequence divergence, taxonomy table, and mapping files were imported into the R tool, the microbiome package was used to analyze the samples.

4.3.6.8.6 Statistical analysis

Mean, standard deviation and standard error were calculated for the duplicated and triplicated values. One way ANOVA with post-hoc Tukey test was applied to determine significant difference between the groups.

4.3.7 Whole-genome analysis of the strains of MSPC

4.3.7.1 DNA extraction

The pure bacterial colony from the agar plate was grown overnight on MRS/TS broth followed by DNA extraction using the InstaGene™ Matrix (Bio-Rad Laboratories, Inc, NY, USA). Further, Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) was used to quantify the DNA.

4.3.7.2 DNA library preparation and sequencing

DNA libraries for high-throughput sequencing were made by commercially available Vazyme TruePrep™ DNA Library Prep Kit V2 for Illumina (Vazyme Biotech, Nanjing, China). The sequencing of the sample libraries was performed on an Illumina HiSeq-2000 sequencing platform (BGI-Shenzhen, Shenzhen, China).

4.3.7.3 Bioinformatics analysis

4.3.7.3.1 Trimming, quality control, and de novo assembly

The trimming of the sequencing reads was performed using the Trimmomatic version 0.38 (Bolger *et al.*, 2014). The contaminated reads were filtered using BWA aligned to the reference sequence. The assembly of the high-quality sequencing reads was performed using Velvet v. 1.2.10 assembler. The de novo assembly results were evaluated using the Quast software. (Gurevich *et al.*, 2013).

4.3.7.3.2 Genome annotation

The assembled genome annotation was performed through the Prokaryotic Genome Annotation Pipeline (PGAP). The Subsystem (a set of functional roles that together implement a specific biological process or structural complex) identification in the genomes and function assigning was determined through online rapid annotation using subsystem technology (RAST) server (Overbeek *et al.*, 2014). The evaluation of orthologous genes was performed via clusters of orthologous genes (COGs) (Galperin *et al.*, 2015) and eggNOG (Huerta-Cepas *et al.*, 2016). Clusters of Orthologous Groups of proteins (COGs) is a data base that phylogenetically classifies the proteins encoded in complete genomes based on similarities in at least three genomes of the same species. The eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) is a database of biological information based on the original idea of COGs and expands to non-supervised orthologous groups constructed from numerous organisms.

4.3.7.3.3 Genome Mining

The genome was mined for the presence of Bacteriocin genes through the online tool BAGEL4 http://bagel4.molgenrug.nl with default settings. For the presence of antibiotic resistance genes in the genomes of the probiotic isolates, online server of CARD https://card.mcmaster.ca with default settings was used (Alcock *et al.*, 2020). To detect prophages in the genomes of the probiotic isolates, PHASTER https://phaster.ca web with default setting was used (Arndt *et al.*, 2016; Zhou *et al.*, 2011). To detect pathogenicity and virulence genes, an online server Islandviewer4

https://www.pathogenomics.sfu.ca/islandviewer with default setting was used (Bertelli *et al.*, 2017). Finally, CRISPR Finder server https://crispr.i2bc.paris-saclay.fr/Server was used to detect genes for the CRISPR or CRISPR Cas (Grissa *et al.*, 2007a; Grissa *et al.*, 2007b, 2008).

4.4 Results

4.4.1 Growth of MSPCs

When both the probiotic communities were checked and compared for their growth on TSA and MSA media. It was observed that the MSPC-2 have comparatively more growth on both the tested media. Significant difference between the growth patterns was observed on Skim Milk media Figure 4.2.

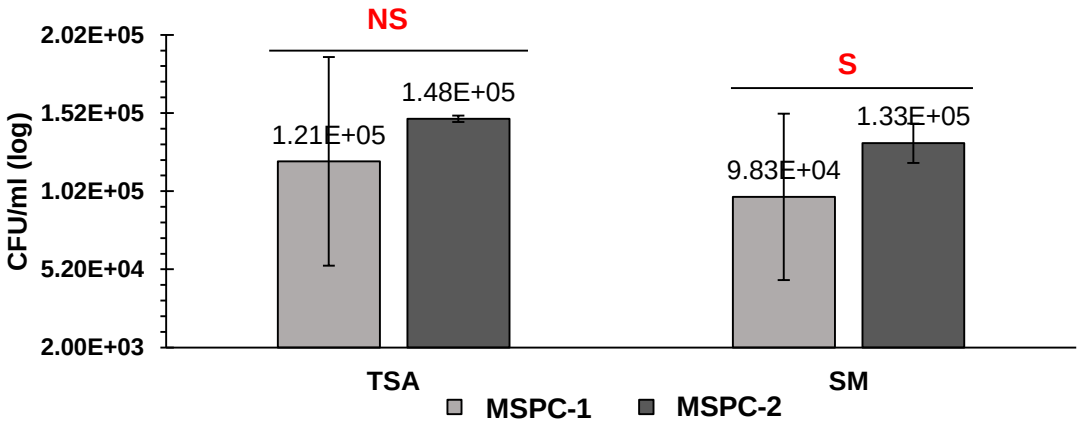

Figure 4. 2: Comparative growth of MSPCs on Tryptic soy and skim milk media after 24 hours of incubation at 37°C (NS = Non-significant, S = significant, based on p-value ≤ 0.05)

4.4.2 The *in vitro* cell surface hydrophobicity and auto-aggregation

The *in vitro* cell surface hydrophobicity and auto-aggregation of MSPCs were evaluated and compared statically. The MSPC-1 had 97.5% of *in vitro* cell surface hydrophobicity and MSPC-2 had 98.5% of *in vitro* cell surface hydrophobicity. But there was no significant difference between cell surface hydrophobicities among the two probiotic communities. Similarly, the MSPC-1 had 62.5% *in vitro* auto-aggregation and MSPC-2 had 83.5% of *in vitro* auto-aggregation. Significant difference is observed between auto-

aggregating abilities between the two-probiotic communities. The results are presented in Figure 4.3.

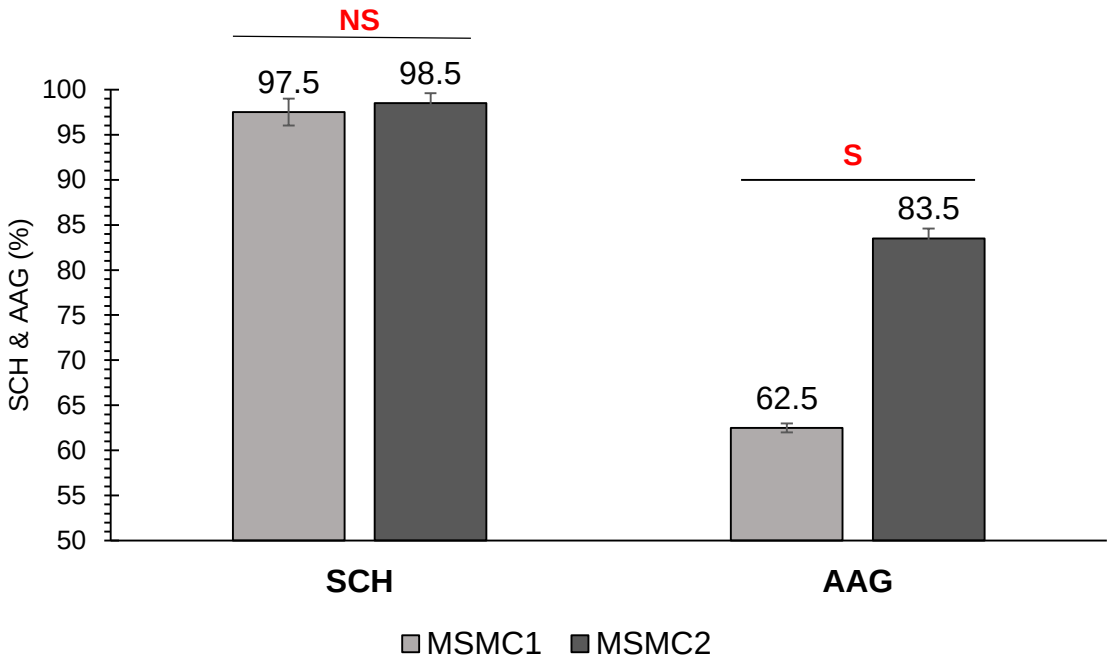

Figure 4. 3: The *in vitro* cell surface hydrophobicity and auto-aggregation of MSPCs (NS = Non-significant, S = significant, based on p- value ≤ 0.05)

4.4.3 Antibacterial effect of MSPCs

The MSPCs showed an antipathogenic effect against ATCC pathogenic strains. The MSPC-1 showed 35.4% inhibition of *B. subtilis* while MSPC-2 showed significant high reduction of *B. subtilis* such as 85.4% (*p-value* ≥ 0.05). No antagonistic effect is observed against *E. coli* by MSPC-1 while MSPC-2 reduced the growth of *E. coli* up to 83. Both the MSPCs inhibited *P. aeruginosa* by 90 and 80% but the difference is not significant. Similarly, MSPC-1 had no activity against *S. enterica* while MSPC-2 inhibited its growth up to 80%. The growth *S. aureus* was affected up to 80% and 88% by MSPC-1 and MSPC-2 respectively but the difference is non-significant. The MSPC-1 inhibited the growth of *S. pneumoniae* up to 62% and the MSPC-2 inhibited the *P. aeruginosa* up to 82% that is significantly high based on *p-value* ≥ 0.05. Results of antibacterial activity by MSPCs are presented in Figure 4.4.

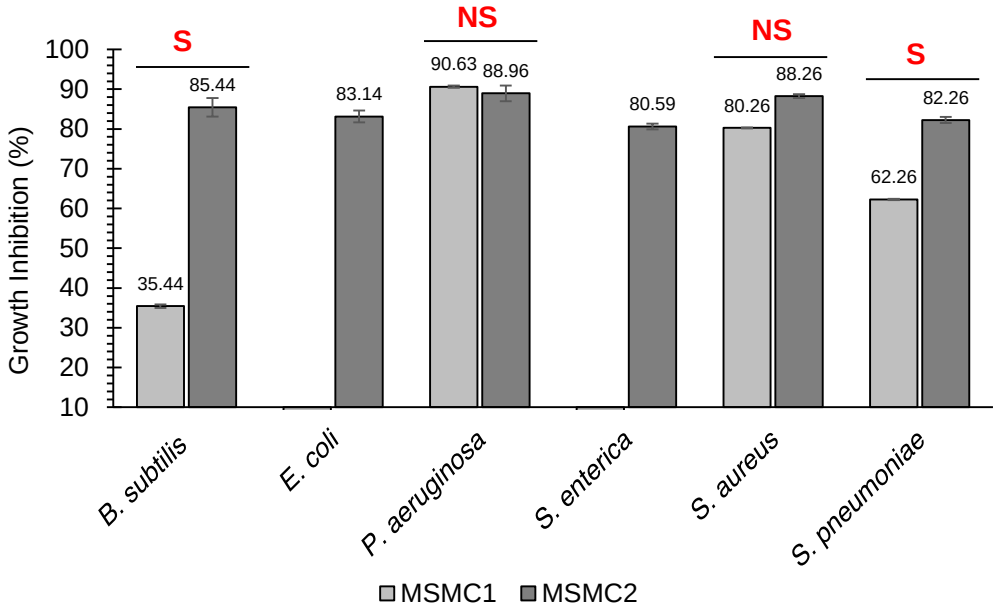

Figure 4. 4: Antibacterial effect of MSPCs of against ATCC pathogens at 37ºC (NS = Non-significant, S = significant, based on p- value ≤ 0.05)

4.4.4 The *in vitro* cholesterol assimilation

The MSPC-1 assimilated 83.1% of cholesterol and MSPC-2 assimilated 87.5% of cholesterol that were provided in the media. Deference between *in vitro* cholesterol assimilation among the two probiotic communities was non-significant.

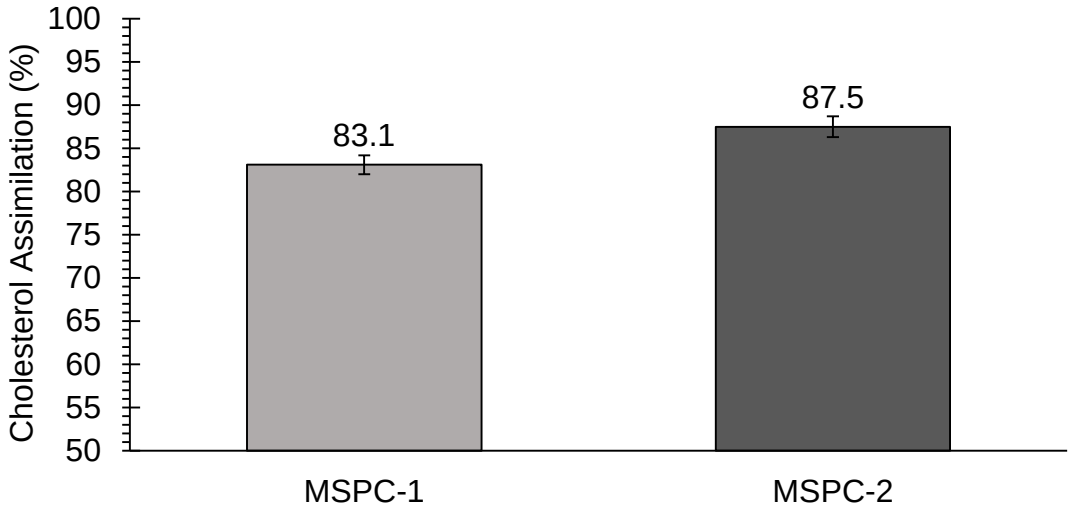

Figure 4. 5: the in vitro cholesterol assimilation of MSPCs after 24 hours of incubation at 37°C (NS = Non-significant, S = significant, based on p- value =/≤ 0.05)

4.4.5 Survival of MSPC-2 in TSI *in vitro* simulated model

4.4.5.1 CFU based Survival

The MSPC-2 was selected from the two communities and was evaluated for its growth in the simulated intestinal (TSI) model. The CFU based survival of MSPC-2 was evaluated in the TSI model, at fasting state, before introducing to the Model the MSPC-2 had 1.4×10^{04} colonies/ml, that reduced to 5×10^{03} CFU/ml in the Stomach at the end of the Duodenum phase their number raised to 1.5×10^{04} CFU/ml, at the end of Jejunum phase their number increased to 1.55×10^{04} CFU/ml and at the end of Ileum phase they retained their initial number 1.4×10^{04} CFU/ml.

At feeding state, before introducing to the Model, the MSPC-2 had 1.8×10^{04} colonies/ml, which reduced to 3×10^{03} CFU/ml in the Stomach; at the end of the Duodenum phase their number raised to 1.5×10^{04} CFU/ml, at the end of Jejunum phase their number increased to 1.2×10^{04} CFU/ml and at the end of Ileum phase they retained to 1.6×10^{04} CFU/ml.

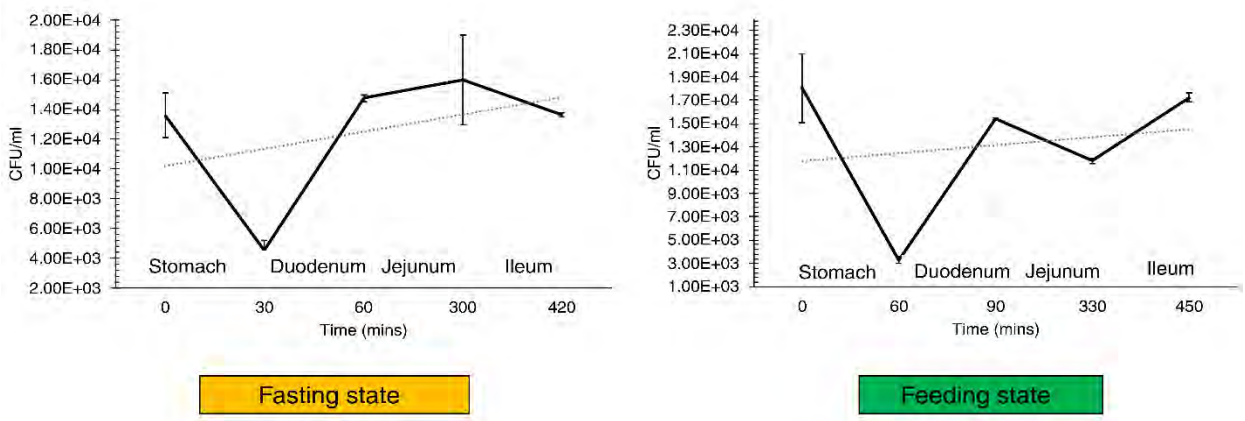

Figure 4. 6: CFU-based survival of MSPC-2 in TSI simulated *in vitro* model during fasting and feeding phases.

4.4.5.2 16S rDNA base survival

During fasting state, before introducing to the TSI model, the *Lactobacillus* had mean OTUs of 20000/ml, during the stomach phase, these reduced to 7338 OTUs/ml, at duodenum phase their number again raised to 20640 OTUs/ml, at jejunum phase the OTUs count decreased to 10228 OTUs/ml and at ileum, the OTUs reached to 20440 OTUs/ml. Before introducing to the TSI model, the *Streptococcus* had mean OTUs of 17 OTUs/ml, during the stomach phase, these increased to 165 OTUs/ml, at duodenum phase, their number again raised to 365 OTUs/ml, at jejunum phase, the OTUs count decreased to 171 OTUs/ml, and at ileum, the OTUs reached to 440 OTUs/ml. Before introducing to the TSI model, the *Lactococcus* had mean OTUs of 407/ml, during the stomach phase, these increased to 875 OTUs/ml, at duodenum phase, their number again raised to 1545 OTUs/ml, at jejunum phase, the OTUs count decreased to 651 OTUs/ml, and at ileum, the OTUs reached to 1400 OTUs/ml. Before introducing to the TSI model, the *Enterococcus* had mean OTUs of 1537/ml, during the stomach phase, these reduced to 447 OTUs/ml, at duodenum phase, their number was 441 OTUs/ml, at jejunum phase, the OTUs count increased to 854 OTUs/ml, and at ileum, the OTUs reached to 3620 OTUs/ml. Results are presented in Figure 4.7.

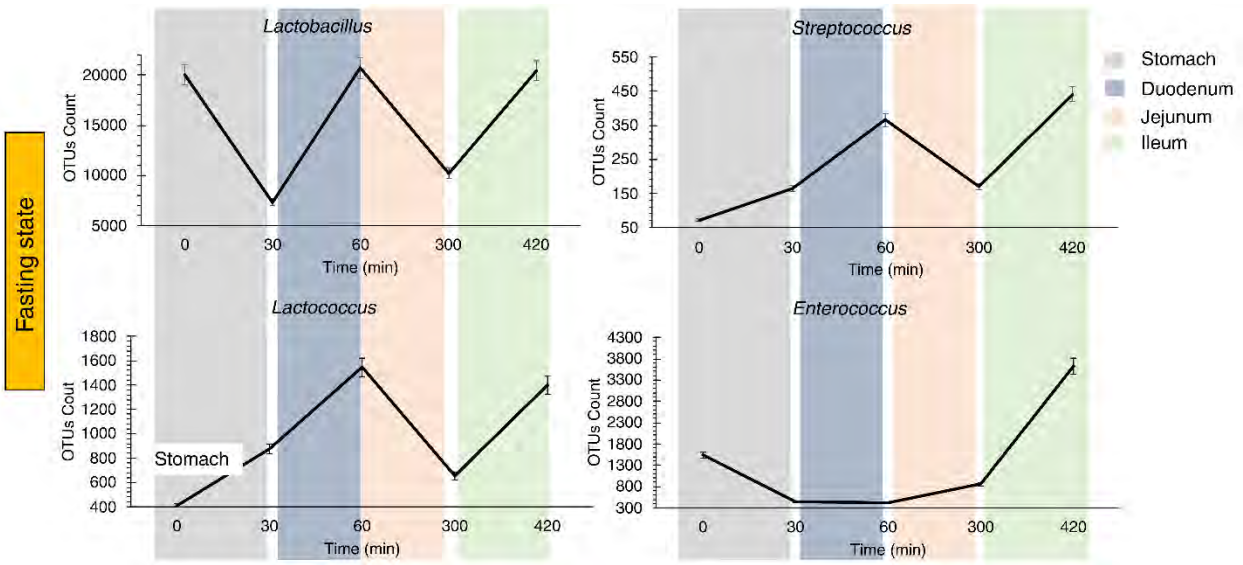

Figure 4. 7: 16S rDNA-based survival of MSPC-2 in TSI *in vitro* simulated model during the fasting phase.

During feeding state, before introducing to the TSI model, the *Lactobacillus* had mean OTUs of 576/ml, during the stomach phase, these reduced to 2015 OTUs/ml, at duodenum phase their number again raised to 80330 OTUs/ml, at jejunum phase the OTUs count decreased to 48952 OTUs/ml and at ileum, the OTUs reached to 33280 OTUs/ml. Before introducing to the TSI model, the *Streptococcus* had mean OTUs of 354 OTUs/ml, during the stomach phase, these were 335 OTUs/ml, at duodenum phase, their number again raised to 500 OTUs/ml, at jejunum phase, the OTUs count decreased to 768 OTUs/ml, and at ileum, the OTUs reached to 1880 OTUs/ml. Before introducing to the TSI model, the *Lactococcus* had mean OTUs of 69/ml, during the stomach phase, these were 90 OTUs/ml, at duodenum phase, their number again raised to 325 OTUs/ml, at jejunum phase, the OTUs count decreased to 116 OTUs/ml, and at ileum, the OTUs reached to 416 OTUs/ml. Before introducing to the TSI model, the *Enterococcus* had mean OTUs of 402/ml, during the stomach phase, these reduced to 290 OTUs/ml, at duodenum phase, their number was 1125 OTUs/ml, at jejunum phase, the OTUs count increased to 3864 OTUs/ml, and at ileum, the OTUs reached to 17090 OTUs/ml. Results are presented in Figure 4.8.

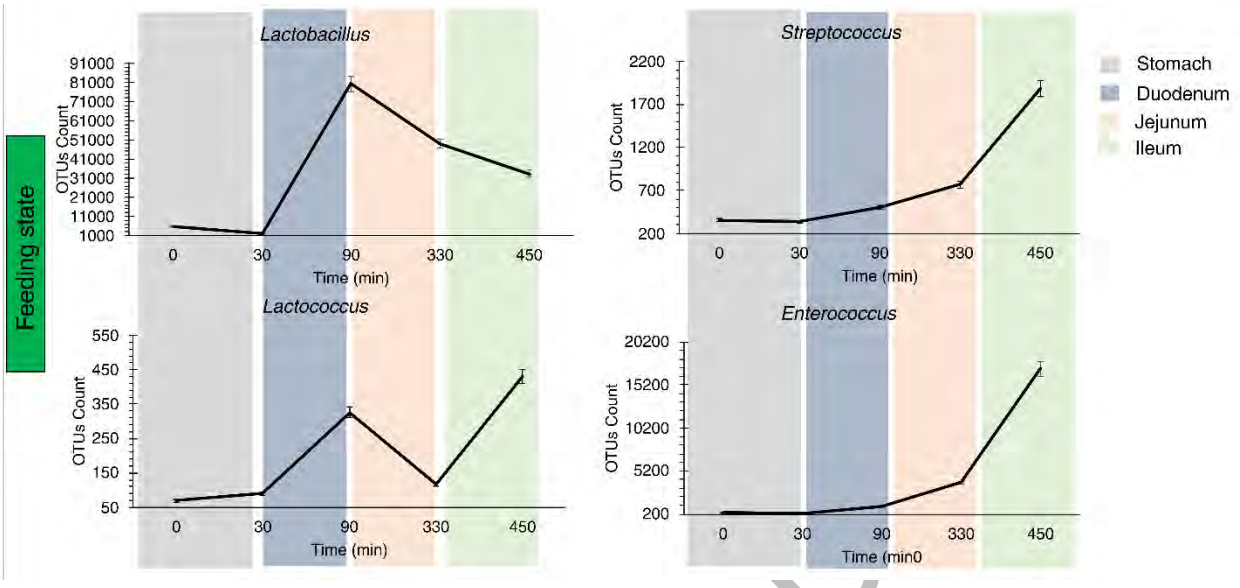

Figure 4. 8: 16S rDNA-based survival of MSPC-2 in TSI *in vitro* simulated model during feeding phase

4.3.4 Genome statistics of the MSPC-2

The size of MPSC-2 Strains' genomes ranged from 1.83 Mb to 3.14 Mb, with an average size of 2.30 Mb. The percentage of GC content ranged from 34.6% to 52.8%, with an average of 44%. The number of contigs ranged from a minimum of 4 to a maximum of 391 with an average of 124 contigs. The number of coding sequences ranged from 1877 to 3248. The detailed results are given in Table 4.1.

Table 4. 1: Genome statistics of the genomes from MSPC-2.

LAB	Genome Size (Mb)	Contigs	GC content %	Total Genes	Coding Sequences	Proteins	Pseudo Genes	RNAs
E. faecium QAUEFNA17	3.10	88	37.5	3112	3021	2888	133	91
E. faecium QAUEFNA13	2.67	81	38.2	2645	2566	2467	79	99
E. faecium QAUEFNN2	2.95	164	37.8	2984	2917	2831	86	67
E. lactis QAUELNN14	2.62	61	38.3	2250	2489	2412	77	81
E. faecium QAUEFNN4	3.05	32	38.0	3027	2947	2840	107	80
E. faecium QAUEFNS1	2.92	192	37.6	2898	2835	2743	92	63
L. acidophilus QAULAN51	1.95	22	34.6	1904	1841	1759	82	63
L. delbrueckii QAULDN14	1.99	156	49.3	2117	2020	1892	128	97
L. delbrueckii QAULDN61	1.83	45	49.8	1986	1892	1738	154	94
L. fermentum QAULFN21	1.86	145	52.8	1848	1785	1724	61	63
L. fermentum QAULFN53	2.07	171	51.7	2148	2082	2002	80	66
L. fermentum QAULFN54	1.96	445	52.0	2059	2039	1965	74	20
L. fermentum QAULFN55	2.01	190	51.8	2041	2005	1935	70	36
L. fermentum QAULFN56	1.90	148	52.4	1919	1867	1803	64	52
L. fermentum QAULFN62	1.96	100	52.0	2001	1941	1869	72	60
L. fermentum QAULFN64	1.90	148	52.4	1919	1867	1803	64	52
L. paracasae QAULPN3	1.93	42	34.7	1858	1818	1755	63	40
L. reutori QAULRN15	2.15	397	38.6	2304	2230	2108	122	74
L. reutori QAULRN18	2.27	194	38.2	2375	2291	2248	43	84
L. rhamnosus QAULRN2	2.91	142	46.7	2847	2770	2734	36	77
S. thermophilus QAUSTN63	1.83	47	38.8	1960	1886	1885	201	74
L. lactis QAULLNA8	2.55	186	35.0	2675	2614	2548	66	61

4.4.6 Subsystem features/genes

The highest number of features (genes) based on SEED subsystems was counted for Carbohydrate metabolism that was 136, then for protein metabolism (124), then for amino acids and derivatives (123). In contrast, minimum features were present for the metabolism of aromatic compounds that counted 1. Detailed results of subsystem features are presented in Figures 4.9.

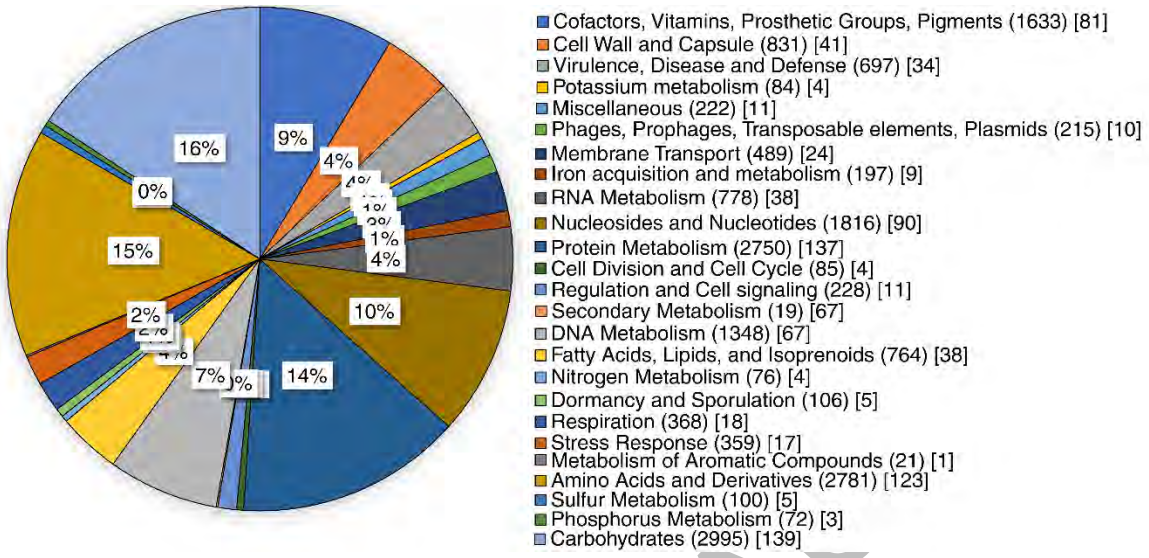

Figure 4. 9: RAST-based subsystem features of the genomes of MSPC-2 (values in parenthesis present collective number of features and values in square brackets present mean values).

Table 4. 2: Subsystem features of MSPMC genomes annotated by RAST

Subsystem features	LAB isolates																					
	E. faecium QAUEFNA17	*E. faecium* QAUEFNA13	*E. faecium* QAUEFNN2	*E. lactis* QAUELNN14	*E. faecium* QAUEFNN4	*E. faecium* QAUEFNS1	*L. acidophilus* QAULAN51	*L. delbrueckii* QAULDN14	*L. delbrueckii* QAULDN61	*L. fermentum* QAULFN21	*L. fermentum* QAULFN53	*L. fermentum* QAULFN54	*L. fermentum* QAULFN55	*L. fermentum* QAULFN56	*L. fermentum* QAULFN62	*L. fermentum* QAULFN64	*L. paracasae* QAULPN3	*L. reutori* QAULRN15	*L. reutori* QAULRN18	*L. rhamnosus* QAULRN2	*S. thermophilus* QAUSTN63	*L. lactis* QAULLNA8
Cofactors, Vitamins, Prosthetic Groups, Pigments	65	58	70	61	62	55	46	59	58	88	92	99	95	92	94	92	46	87	82	48	80	104
Cell Wall and Capsule	51	51	51	48	49	46	29	45	36	24	24	26	20	24	25	24	42	34	31	37	59	55
Virulence, Disease and Defense	47	40	44	36	44	43	33	20	20	25	34	30	30	23	22	23	30	34	30	26	21	42
Potassium metabolism	4	3	3	3	4	4	5	3	3	4	3	3	5	3	3	3	5	3	4	4	2	10
Miscellaneous	7	10	7	8	8	7	7	7	7	12	13	11	13	10	14	10	9	14	10	17	8	13
Phages, Prophages, Transposable elements, Plasmids	7	1	16	9	15	6	0	0	6	1	6	6	13	3	1	3	0	42	12	21	0	47
Membrane Transport	29	36	30	32	27	31	23	3	32	11	11	11	16	8	9	8	25	28	22	23	40	34
Iron acquisition and metabolism	14	13	13	22	12	13	4	6	4	8	8	9	8	8	9	8	4	5	7	6	11	5
RNA Metabolism	41	36	41	38	38	39	31	34	34	35	35	36	35	34	35	34	32	36	36	35	31	32
Nucleosides and Nucleotides	86	76	82	75	82	82	80	76	81	87	89	86	86	89	88	89	69	77	92	83	84	77
Protein Metabolism	182	167	165	163	170	164	114	104	79	108	111	89	93	107	106	107	110	130	134	129	111	107
Cell Division and Cell Cycle	3	3	3	4	3	3	4	4	4	4	4	4	4	4	4	4	4	5	4	5	4	4
Regulation and Cell signaling	20	16	15	14	15	15	8	10	12	4	4	6	4	4	4	4	7	8	11	25	9	13
Secondary Metabolism	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	2	1	4	6	4
DNA Metabolism	74	70	76	59	69	78	45	67	53	46	60	60	61	44	59	44	55	54	62	62	92	58
Fatty Acids, Lipids, and Isoprenoids	27	27	27	27	27	27	23	34	36	42	42	46	45	43	44	43	27	41	28	43	21	44
Nitrogen Metabolism	0	0	0	0	0	0	0	0	0	9	9	9	15	0	9	0	0	8	8	4	0	5
Dormancy and Sporulation	6	6	6	8	6	0	6	5	5	5	5	5	5	5	5	5	5	5	5	6	1	1
Respiration	23	23	22	22	23	24	12	10	10	15	15	15	14	13	14	13	13	15	15	27	11	19
Stress Response	29	30	30	30	29	25	5	10	9	12	15	15	11	10	12	10	6	8	8	21	9	25
Metabolism of Aromatic Compounds	1	1	1	1	1	1	0	4	0	0	0	0	0	0	0	0	2	2	2	1	2	2
Amino Acids and Derivatives	134	145	136	145	138	131	86	101	97	141	134	124	145	127	138	125	37	120	122	104	169	182
Sulfur Metabolism	2	3	2	3	2	2	4	3	3	4	3	3	3	3	3	3	3	2	3	40	3	3
Phosphorus Metabolism	3	2	3	3	3	3	0	6	6	5	4	4	4	4	4	4	0	4	4	3	0	3
Carbohydrates	194	155	196	159	191	198	95	82	72	105	124	129	115	120	120	120	92	113	95	232	113	175
Total Features	1049	972	1039	970	1018	997	661	693	667	795	845	826	840	778	822	776	624	877	828	1006	887	1064

4.3.5 EggNOG COGs

Based on the EggNOG database, the highest COGs features were counted 181 responsible for replication, recombination, and repair, then 157 were features responsible for Transcription, 157 were responsible for translation, ribosomal structure, and biogenesis. Four hundred eight features had no known functions. Detailed of EggNOG based COGs results are presented in Figure 4.10.

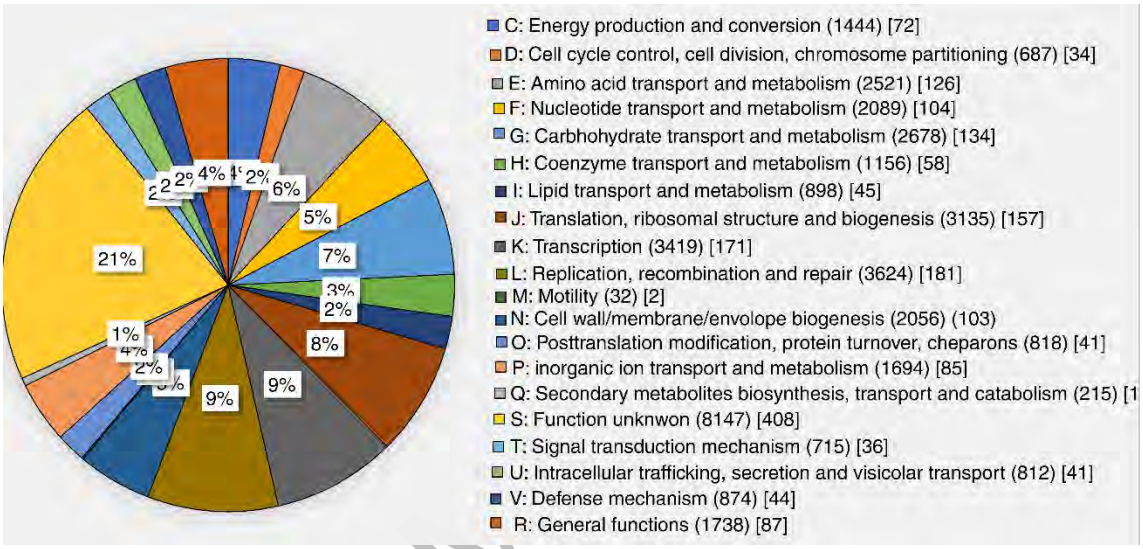

Figure 4. 10: COGs feature of genomes isolated from MSPC-2 according to the EggNOG database (values in parenthesis present collective number of features and values in square brackets present mean values)

4.3.6 Bacteriocin genes

When the genomes were mined for Bacteriocin genes, 13 different types of bacteriocin genes were present in the genomes of MSPC-2. Detailed results are presented in Table 4.

Table 4. 3: EggNOG based COGs of MSPMC isolated from traditional fermented milk product Dahi

EggNOG features	*E. faecium* QAUEFNA17	*E. faecium* QAUEFNA13	*E. faecium* OAUEFNN2	*E. lactis* OAUELNN14	*E. faecium* OAUEFNN4	*E. faecium* OAUEFNS1	*L. acidophilus* OAULAN51	*L. delbrueckii* QAULDN14	*L. delbrueckii* OAULDN61	*L. fermentum* OAULFN21	*L. fermentum* OAULFN53	*L. fermentum* OAULFN54	*L. fermentum* QAULFN55	*L. fermentum* OAULFN56	*L. fermentum* OAULFN62	*L. fermentum* OAULFN64	*L. paracasae* QAULPN3	*L. reutori* QAULRN15	*L. reutori* OAULRN18	*L. rhamnosus* QAULRN2	*S. thermophilus* QAUSTN63	*L. lactis* QAULLNA8
C: Energy production and conversion	69	68	72	69	71	73	68	44	48	75	84	81	85	72	84	72	53	75	71	90	89	57
D: Cell cycle control, cell division, chromosome partitioning	40	36	43	32	38	46	32	31	27	31	34	35	32	31	29	31	31	31	32	42	35	31
E: Amino acid transport and metabolism	123	126	128	125	127	124	97	120	130	135	144	129	155	125	140	125	82	127	107	136	141	155
F: Nucleotide transport and metabolism	91	91	93	91	93	92	104	97	105	111	114	113	110	108	113	108	93	103	104	113	133	91
G: Carbohydrate transport and metabolism	240	211	254	250	262	248	124	83	69	68	69	69	75	89	69	89	146	82	79	237	115	80
H: Coenzyme transport and metabolism	47	47	52	16	47	51	34	40	40	70	81	84	77	70	76	70	33	72	64	55	46	51
I: Lipid transport and metabolism	50	50	51	48	51	50	34	40	40	47	48	53	45	44	49	48	36	42	37	46	37	30
J: Translation, ribosomal structure and biogenesis	169	162	166	162	165	168	152	142	151	152	151	152	153	151	150	153	154	160	150	168	166	158
K: Transcription	247	282	246	218	240	240	139	125	115	127	138	142	138	136	137	136	139	140	135	226	191	104
L: Replication, recombination, and repair	337	155	228	128	301	213	116	272	165	131	205	136	134	133	153	133	113	188	223	148	140	207
M: Cell wall/membrane/envelope biogenesis	145	116	138	115	117	138	94	91	80	66	85	82	83	81	87	81	100	122	112	122	116	102
N: Motility	0	3	1	4		1	1	3	2	1	2	3	1	1	1	1	5	2	4	0	0	
O: Posttranslational modification, protein turnover, chaperons	47	53	49	45	46	49	36	34	36	37	41	40	39	38	40	37	36	35	40	49	36	40
P: Inorganic ion transport and metabolism	100	106	96	91	93	92	90	77	75	75	78	74	81	69	70	69	86	74	53	106	130	102
Q: Secondary metabolites biosynthesis, transport, and catabolism	15	16	15	22	14	15	6	6	5	8	11	10	9	10	9	10	6	11	9	16	14	12
S: Function unknown	594	511	582	522	582	570	347	279	311	294	330	324	344	318	296	318	358	438	346	509	496	291
T: Signal transduction mechanism	50	39	47	42	48	48	27	66	44	26	29	25	27	22	26	23	28	31	33	43	33	24
U: Intracellular trafficking, secretion, and vesicular transport	62	66	65	62	66	64	30	24	24	36	37	39	36	32	36	32	32	32	34	36	29	22
V: Defense mechanism	58	53	56	51	56	55	44	61	60	19	28	33	29	20	28	20	54	28	31	86	55	66
R: General functions	80	101	99	94	101	95	59	73	83	85	92	92	94	103	91	91	53	86	68	101	91	80
Total Features	2564	2292	2481	2187	2518	2432	1633	1706	1611	1595	1800	1715	1749	1653	1684	1647	1634	1882	1730	2333	2093	1703

Table 4. 4: The detail of Bacteriocin genes present in the genomes of MSPC-2.

AOI	Class	Position			Bacteria
		Contig	Start	End	
NODE_1.4.AOI_01	107.2; Hiracin_JM79	NODE_1	776570	796708	*E. faecium* QAUEFNA17
NODE_5.10.AOI_01	15.2; Bac43	NODE_5	1	6249	*E. faecium* QAUEFNN4
NODE_5.74.AOI_01	15.2; Bac43	NODE_5	1	6249	*E. faecium* QAUEFNS1
NODE_10.14.AOI_01	21.2; bacteriocin_LS2chainb	NODE_10	1	17686	*L. paracasae* QAULPN3
NODE_51.0.AOI_01	231.2; BlpD	NODE_51	5900	19028	*S. thermophilus* QAUSTN63
NODE_06.26.AOI_01	295.1; Streptide	NODE_06	255932	276019	*S. thermophilus* QAUSTN68
NODE_90.0.AOI_01	43.2; Bovicin_255_variant	NODE_90	45521	65782	*L. delbrueckii* QAULDN14
NODE_64.13.AOI_01	51.2; Carnocin_CP52	NODE_64	90572	116422	*L. rhamnosus* QAULRN2
NODE_17.5.AOI_01	6.2;Acidocin_J1132_beta_peptide	NODE_17	47011	72774	*L. acidophilus* QAULAN51
NODE_20.9.AOI_01	6.3; Bacteriocin_helveticin_J	NODE_20	72314	86213	*L. acidophilus* QAULAN51
NODE_36.17.AOI_01	6.3; Bacteriocin_helveticin_J	NODE_36	77630	98608	*L. paracasae* QAULPN3
NODE_5.7.AOI_01	62.3; enterolysin_A	NODE_5	29303	49837	*E. faecium* QAUEFNA17
NODE_95.68.AOI_01	62.3; enterolysin_A	NODE_95	0	16993	*E. faecium* QAUEFNS1
NODE_1.20.AOI_01	62.3; enterolysin_A	NODE_1	46971	67430	*L. fermentum* QAULFN53
NODE_107.13.AOI_01	62.3; enterolysin_A	NODE_107	38328	58769	*L. fermentum* QAULFN55
NODE_17.52.AOI_01	62.3; enterolysin_A	NODE_17	3618	24059	*L. fermentum* QAULFN56
NODE_17.52.AOI_01	62.3; enterolysin_A	NODE_17	3618	24059	*L. fermentum* QAULFN64
NODE_338.77.AOI_01	62.3; enterolysin_A	NODE_338	0	10778	*L. reutori* QAULRN15
NODE_14.2.AOI_02	63.3; Enterolysin_A	NODE_14	9673	30216	*E. faecium* QAUEFNA13
NODE_75.66.AOI_01	63.3; Enterolysin_A	NODE_75	0	16931	*E. faecium* QAUEFNN2
NODE_95.2.AOI_01	63.3; Enterolysin_A	NODE_95	0	16993	*E. faecium* QAUEFNN4
NODE_190.40.AOI_01	63.3; Enterolysin_A	NODE_190	6911	17955	*L. reutori* QAULRN15
NODE_179.73.AOI_01	63.3; Enterolysin_A	NODE_179	0	11994	*L. reutori* QAULRN15
NODE_44.3.AOI_01	63.3; Enterolysin_A	NODE_44	39265	53617	*L. rhamnosus* QAULRN2
NODE_11.4.AOI_01	64.3; Enterolysin_A	NODE_11	85135	105564	*L. acidophilus* QAULAN51
NODE_11.24.AOI_01	64.3; Enterolysin_A	NODE_11	0	17210	*L. delbrueckii* QAULDN61
NODE_22.21.AOI_01	64.3; Enterolysin_A	NODE_22	15407	31836	*L. fermentum* QAULFN62
NODE_322.149.AOI_01	64.3; Enterolysin_A	NODE_322	1814	15695	*L. reutori* QAULRN15
NODE_1.4.AOI_02	81.2; Enterocin_A	NODE_1	333626	353800	*E. faecium* QAUEFNA17
NODE_106.83.AOI_01	81.2; Enterocin_A	NODE_106	0	12149	*E. faecium* QAUEFNN2
NODE_135.36.AOI_01	81.2; Enterocin_A	NODE_135	6347	17165	*E. faecium* QAUEFNN4
NODE_135.67.AOI_01	81.2; Enterocin_A	NODE_135	6347	17165	*E. faecium* QAUEFNS1
NODE_40.47.AOI_01	91.2; Enterocin_P	NODE_40	1	4499	*E. faecium* QAUEFNA13
NODE_14.2.AOI_01	95.2; Enterocin_SE-K4	NODE_14	0	10809	*E. faecium* QAUEFNA13
NODE_07.12.AOI_01	95.2; Enterocin_SE-K4	NODE_07	0	8463	*E. lactis* QAUELNN14
NODE_40.17.AOI_01	Lanthipeptide_class_I	NODE_40	21602	41602	*S. thermophilus* QAUSTN63
NODE_101.78.AOI_01	Lanthipeptide_class_IV	NODE_101	25898	45898	*L. delbrueckii* QAULDN14
NODE_59.15.AOI_01	Sactipeptides	NODE_59	1004	21004	*L. lactis* QAULLNA8
NODE_116.1.AOI_01	Sactipeptides	NODE_116	890	23008	*L. lactis* QAULLNA8
NODE_14.22.AOI_01	Sactipeptides	NODE_14	109085	129085	*S. thermophilus* QAUSTN63
NODE_05.25.AOI_01	Sactipeptides	NODE_05	69089	89089	*S. thermophilus* QAUSTN63
NODE_05.25.AOI_02	Sactipeptides	NODE_05	82721	102721	*S. thermophilus* QAUSTN63
NODE_25.27.AOI_01	Sactipeptides	NODE_25	-7810	12190	*S. thermophilus* QAUSTN643

4.3.7 Antibiotic resistance genes

The genomes of the isolates were mined for the presence of antibiotic resistance genes. It was found that the strains of *E. faecium* had a high number of antibiotic resistance genes. The *E. faecium* QAUEFNA17 and *E. faecium* QAUEFNS1 have 15 antibiotic resistance genes. The *E. faecium* QAUEFNN2 has 14 resistance genes. While the *L. lactis* QAULLNA8, *L. rhamnosus* QAULRN2, and *L. delreuckii* QAULDN61 had one antibiotic resistance gene. The remaining strains had no antibiotic resistance gene. The detail of antibiotic resistance genes is presented in Table 4.5.

Table 4. 5: Detail of the antibiotic resistance genes of the isolates mined through CARD database.

***E. faecium* QAUEFNA17**			
ARO Term	AMR Family	Drug Class	Resistance Mechanism
efmA	MFS antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic	Antibiotic efflux
APH(3')-IIIa	APH(3')	Aminoglycoside antibiotic	Antibiotic inactivation
vanZA	vanZ, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanHA	vanH, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanRA	glycopeptide resistance gene cluster, vanR	Glycopeptide antibiotic	Antibiotic target alteration
APH(3')-IIIa	APH(3')	Aminoglycoside antibiotic	Antibiotic inactivation
AAC(6')-Ii	AAC(6')	Aminoglycoside antibiotic	Antibiotic inactivation
AAC(6')-Ie-APH(2'')-Ia	APH(2''), AAC(6')	Aminoglycoside antibiotic	Antibiotic inactivation
aad(6)	ANT(6)	Aminoglycoside antibiotic	Antibiotic inactivation
ErmB	Erm 23S ribosomal RNA methyltransferase	Macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	Antibiotic target alteration
vanYA	vanY, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanXA	vanX, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanA	Glycopeptide resistance gene cluster, van ligase	Glycopeptide antibiotic	Antibiotic target alteration
aad(6)	ANT(6)	Aminoglycoside antibiotic	Antibiotic inactivation
ErmB	Erm 23S ribosomal RNA methyltransferase	Macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	Antibiotic target alteration

***E. faecium* QAUEFNN2**			
efmA	Major facilitator superfamily antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic	Antibiotic efflux
APH(3')-IIIa	APH(3')	Aminoglycoside antibiotic	Antibiotic inactivation
dfrF	Trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine antibiotic	Antibiotic target replacement
aad(6)	ANT(6)	Aminoglycoside antibiotic	Antibiotic inactivation
AAC(6')-Ii	AAC(6')	Aminoglycoside antibiotic	Antibiotic inactivation
dfrG	Trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine antibiotic	Antibiotic target replacement
dfrG	Trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine antibiotic	Antibiotic target replacement
AAC(6')-Ii	AAC(6')	Aminoglycoside antibiotic	Antibiotic inactivation
tet(W/N/W)	Tetracycline-resistant ribosomal protection protein	Tetracycline antibiotic	Antibiotic target protection
tetM	Tetracycline-resistant ribosomal protection protein	Tetracycline antibiotic	Antibiotic target protection
tet(45)	Major facilitator superfamily antibiotic efflux pump	Tetracycline antibiotic	Antibiotic efflux
ErmT	Erm 23S ribosomal RNA methyltransferase	Macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	Antibiotic target alteration
AAC(6')-Ie-APH(2'')-Ia	APH(2''), AAC(6')	Aminoglycoside antibiotic	Antibiotic inactivation
E. faecium liaR mutant	Daptomycin resistant liar	Peptide antibiotic	Antibiotic target alteration, antibiotic efflux
E. faecium liaS mutant	Daptomycin resistant liaS	Peptide antibiotic	Antibiotic target alteration, antibiotic efflux

***E. faecium* QAUEFNS1**			
vanYA	vanY, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanZA	vanZ, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanHA	vanH, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanRA	Glycopeptide resistance gene cluster, vanR	Glycopeptide antibiotic	Antibiotic target alteration
efmA	Major facilitator superfamily antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic	Antibiotic efflux
APH(3')-IIIa	APH(3')	Aminoglycoside antibiotic	Antibiotic inactivation
vanA	Glycopeptide resistance gene cluster, van ligase	Glycopeptide antibiotic	Antibiotic target alteration
vanXA	vanX, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
vanSA	vanS, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration
dfrF	Trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine antibiotic	Antibiotic target replacement
tetM	Tetracycline-resistant ribosomal protection protein	Tetracycline antibiotic	Antibiotic target protection
AAC(6')-Ii	AAC(6')	Aminoglycoside antibiotic	Antibiotic inactivation
ErmB	Erm 23S ribosomal RNA methyltransferase	Macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	Antibiotic target alteration
aad(6)	ANT(6)	Aminoglycoside antibiotic	Antibiotic inactivation
SAT-4	Streptothricin acetyltransferase (SAT)	Nucleoside antibiotic	Antibiotic inactivation

***L. Lactis* QAULLNA8**			
lmrD	ATP-binding cassette (ABC) antibiotic efflux pump	Lincosamide antibiotic	Antibiotic efflux

***L. rhamnosus* QAULRN2**			
TEM-116	TEM beta-lactamase	Monobactam, cephalosporin, penam	Antibiotic inactivation

***L. delbrueckii* QAULD61**			
Trimethoprim-resistant dihydrofolate reductase DfrA42	trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine antibiotic	Antibiotic target replacement

4.3.8 Phage genes in the genomes of MSPC-2 isolates

The genomes of the isolates were mined for the presence of the Prophages. It detected intact, incomplete, or questionable Prophages. The *E. faecium* QAUEFNN4 had 11 prophages, of which 3 were intact, 4 were incomplete, and 4 were questionable. The *L. reutori* QAULRN15 had 9 prophages, 3 were intact, 5 were incomplete, while only 1 is questionable. The *L. fermentum* QAULFN51 had only one incomplete phage. The *L. fermentum* QAULFN53 had only one intact phage. The detail of prophages is presented in Table 4.6.

Table 4. 6: Detail of the prophage genes present in the genomes of MSPC-2 strains.

LAB Isolates	Most Common Phage	Region	Region Position	Completeness
L. lactis QAULLNA8	PHAGE_Lactoc_ul36_NC_004066(10)	NODE_22	196-16059	Incomplete
	PHAGE_Lactoc_bIL309_NC_002668(24)	NODE_28	32000-70420	Intact
	PHAGE_Lactoc_bIL285_NC_002666(21)	NODE_30	6216-39044	Intact
	PHAGE_Lactoc_bIL311_NC_002670(11)	NODE_40	10565-41527	Questionable
	PHAGE_Lactoc_62503_NC_049811(7)	NODE_150	304-30434	Incomplete
L. rhamnosus QAULRN2	PHAGE_Lactob_PL_1_NC_022757(23)	NODE_43	1175-36561	Intact
	PHAGE_Bacill_G_NC_023719(2)	NODE_50	208417-226951	Incomplete
	PHAGE_Entero_IME_EFm5_NC_028826(3)	NODE_63	72641-86389	Questionable
	PHAGE_Lactob_PLE3_NC_031125(29)		167162-202422	Incomplete
L. reutori QAULRN18	PHAGE_Lactob_Lv_1_NC_011801(2)	NODE_14	3123-8066	Incomplete
	PHAGE_Geobac_E3_NC_029073(7)	NODE_108	10623-56283	Incomplete
	PHAGE_Lactob_LfeSau_NC_029068(4)	NODE_141	18979-28679	Questionable
	PHAGE_Strept_7201_NC_002185(7)	NODE_144	10105-43889	Intact
	PHAGE_Lactob_Lj771_NC_010179(3)		30470-61808	Incomplete
	PHAGE_Lactob_LBR48_NC_027990(10)	NODE_149	13783-53412	Intact
L. reutori QAULRN15	PHAGE_Coryne_Lederberg_NC_048790(6)	NODE_1	21716-47756	Incomplete
	PHAGE_Anoxyb_A403_NC_048701(2)	NODE_16	1453-6395	Incomplete
	PHAGE_Strept_DT1_NC_002072(8)	NODE_68	3693-22005	Intact
	PHAGE_Lactob_LF1_NC_019486(15)	NODE_179	557-17930	Intact
	PHAGE_Brocho_BL3_NC_015254(2)	NODE_190	746-14760	Questionable
	PHAGE_Lactob_phig1e_NC_004305(11)	NODE_234	404-16957	Intact
	PHAGE_Lactob_jlb1_NC_024206(3)	NODE_245	363-12846	Incomplete
	PHAGE_Entero_IME_EFm5_NC_028826(3)	NODE_337	31-14143	Incomplete
	PHAGE_Bacill_250_NC_029024(3)	NODE_349	571-12246	Incomplete
L. paracasae QAULPN3	PHAGE_Bacill_G_NC_023719(4)	NODE_12	6728-14152	Incomplete
L. fermentum QAULFN62	PHAGE_Lactob_LF1_NC_019486(3)	NODE_9	761-10052	Incomplete
	PHAGE_Lactob_Lb338_1_NC_012530(2)	NODE_19	26655-35922	Incomplete
	PHAGE_Lactob_LfeSau_NC_029068(18)	NODE_47	16147-38147	Intact

L. fermentum QAULFN55	PHAGE_Lactob_LF1_NC_019486(23)	NODE_107	35570-64064	Intact
L. fermentum QAULFN54	PHAGE_Lactob_LF1_NC_019486(3)	NODE_66	4372-15977	Incomplete
	PHAGE_Geobac_E2_NC_009552(2)	NODE_72	1-11185	Questionable
L. fermentum QAULFN53	PHAGE_Lactob_LF1_NC_019486(20)	NODE_1	31327-108753	Intact
L. fermentum QAULFN21	PHAGE_Nodula_vB_NspS_kac65v151	NODE_63	49861-58332	Incomplete
	PHAGE_Lactob_Lrm1_NC_011104(2)	NODE_73	148940-156114	Incomplete
	PHAGE_Entero_DE3_NC_042057(1)	NODE_74	84400-92188	Incomplete
L. fermentum QAULFN64	PHAGE_Lactob_JCL1032_NC_019456(10)	NODE_17	1217-30263	Intact
	PHAGE_Lactob_LF1_NC_019486(12)	NODE_27	358-21290	Incomplete
	PHAGE_Burkho_BcepSaruman_NC_049850(1)	NODE_37	41315-50947	Incomplete
L. fermentum QAULFN56	PHAGE_Lactob_JCL1032_NC_019456(10)	NODE_17	1217-30263	Intact
	PHAGE_Lactob_LF1_NC_019486(12)	NODE_27	358-21290	Incomplete
	PHAGE_Entero_DE3_NC_042057(1)	NODE_37	41315-50947	Incomplete
L. delbrueckii QAULDN61	PHAGE_Lactob_Lj771_NC_010179(21)	NODE_10	102604-145163	Intact
	PHAGE_Strept_315.2_NC_004585(2)		219342-253941	Incomplete
L. delbrueckii QAULDN14	PHAGE_Lactoc_BK5_T_NC_002796(1)	NODE_75	4512-19743	Incomplete
L. acidophilus QAULAN51	PHAGE_Lactoc_949_NC_015263(1)	NODE_11	522797-534507	Incomplete
E. faecium QAUEFNS1	PHAGE_Entero_phiFL1A_NC_013646(6)	NODE_22	74-41710	Intact
	PHAGE_Clostr_PhiS63_NC_017978(1)	NODE_122	22-26788	Incomplete
	PHAGE_Geobac_E3_NC_029073(2)	NODE_149	7941-13836	Incomplete
E. faecium QAUEFNN4	PHAGE_Paenib_Shelly_NC_041909(2)	NODE_1	45065-68764	Questionable
	PHAGE_Vibrio_vB_VspP_pVa5_NC_049379(1)		340065-349349	Incomplete
	PHAGE_Coryne_Poushou_NC_042139(2)		509456-541611	Incomplete
	PHAGE_Prochl_P_SSM2_NC_006883(2)		1272752-1281907	Incomplete
	PHAGE_Lister_2389_NC_003291(14)		1603641-1643174	Intact
	PHAGE_Mycoba_Nhonho_NC_028815(1)		1856696-1891086	Incomplete
	PHAGE_Entero_EFAP_1_NC_012419(3)		2570737-2585545	Questionable
	PHAGE_Escher_ECBP1_NC_018854(52)		2682689-2754720	Intact
	PHAGE_Staphy_SPbeta_like_NC_029119(4)	NODE_2	66132-92963	Questionable
	PHAGE_Shigel_POCJ13_NC_025434(2)		105617-122123	Questionable
	PHAGE_Staphy_SPbeta_like_NC_029119(6)		178800-200199	Intact
E. faecium QAUEFNN2	PHAGE_Erwini_vB_Ehours_59_NC_048198(1)	NODE_87	13-16177	Incomplete
	PHAGE_Lister_2389_NC_003291(14)	NODE_114	3194-29003	Questionable
	PHAGE_Entero_phiFL1A_NC_013646(6)	NODE_138	16985-47825	Intact
	PHAGE_Bacill_phBC6A52_NC_004821(12)	NODE_148	1-34335	Questionable
	PHAGE_Geobac_E3_NC_029073(2)	NODE_150	2-6387i	Incomplete
E. faecium QAUEFNA17	PHAGE_Lister_*2389*_NC_003291(14)	NODE_1	364719-413204	Intact
	PHAGE_Entero_EFAP_1_NC_012419(3)		621630-636233	Questionable
	PHAGE_Stx2_c_1717_NC_011357(2)		943954-951327	Questionable
	PHAGE_Bacill_BSP38_NC_048726(1)	NODE_2	48005-66369	Incomplete

	PHAGE_Bacter_Diva_NC_028788(2)	NODE_3	12068-34619	Questionable
	PHAGE_Staphy_SPbeta_like_NC_029119(7)		70893-113559	Intact
	PHAGE_Paenib_Xenia_NC_028837(2)		108059-115037	Questionable
	PHAGE_Escher_RCS47_NC_042128(2)	NODE_6	8702-47221	Intact
E. faecium QAUEFN13	PHAGE_Lactob_phiAT3_NC_005893(2)	NODE_24	8900-16918	Incomplete
	PHAGE_Lactob_Lj965_NC_005355(2)	NODE_81	82819-101022	Incomplete
	PHAGE_Bacill_vB_BtS_BMBtp14_NC_048640(2)	NODE_04	76723-84169	Incomplete
	PHAGE_Bacill_phBC6A52_NC_004821(11)	NODE_22	120911-157819	Intact
	PHAGE_Lister_2389_NC_003291(14)	NODE_29	20976-59224	Intact
	PHAGE_Lactob_iLp84_NC_028783(2)		73917-78025	Incomplete
	PHAGE_Escher_520873_NC_049344(2)	NODE_37	96-6158	Incomplete
E. lactis QAUELNN14	PHAGE_Bacill_SPbeta_NC_001884(2)	NODE_17	2599-13048	Incomplete

4.3.9 Pathogenicity islands and virulence genes

The genomes of the isolates were mined for the presence of curated virulence factors, homologous virulence factors, and pathogen associated genes using the Islandviewer4 database. There were no virulent, resistant, or pathogenic genes in any of the mined genomes.

4.3.10 CRISPSR genes in the genomes of the isolates

The genomes of the probiotic isolates were searched for the presence of CRISPR sequences. Except for the genome of *E. faecium* QAUEFNN2, all the genomes had CRISPR sequences. The detail of the CRISPR sequences is given in Table 4.7.

Table 4. 7: Detail of the CRISPR sequences present in the genomes of MSPC-2 strains.

LAB isolate	Crisper ID	Crisper Nature	DR	Spacers	Position		
					Contig no	Start	END
L. lactis QAULLNA8	tmp_49_PossibleCrispr_1	Possible	24	1	NODE_22	14444	14538
	tmp_101_PossibleCrispr_1	Possible	35	1	NODE_43	2719	2868
	tmp_101_PossibleCrispr_2	Possible	35	2	NODE_43	3073	3311
L. rhamnosus QAULRN2	tmp_22_PossibleCrispr_1	Possible	43	1	NODE_22	77078	77223
	tmp_39_PossibleCrispr_1	Possible	51	1	NODE_39	168	319
	tmp_52_PossibleCrispr_1	Possible	24	1	NODE_50	571541	571647
	tmp_105_PossibleCrispr_1	Possible	43	1	NODE_63	147856	147999
	tmp_127_Crispr_1	Confirmed	36	28	NODE_65	71816	73699
L. reutori QAULRN18	tmp_13_PossibleCrispr_1	Possible	26	2	NODE_13	49994	50127
L. reutori QAULRN15	tmp_95_PossibleCrispr_1	Possible	55	1	NODE_95	1105	1254
L. paracasae QAULPN3	tmp_3_Crispr_1	Confirmed	36	43	NODE_3	47672	50545
L. fermentum QAULFN62	tmp_9_Crispr_1	Confirmed	36	16	NODE_9	44217	45309
	tmp_11_Crispr_1	Confirmed	28	67	NODE_11	51208	55326
	tmp_19_Crispr_1	Confirmed	36	7	NODE_19	15915	16454
	tmp_37_PossibleCrispr_1	Possible	37	1	NODE_37	3635	3791
L. fermentum QAULFN55	tmp_129_Crispr_1	Confirmed	29	17	NODE_131	53	1118
L. fermentum QAULFN53	tmp_1_PossibleCrispr_1	Possible	23	1	NODE_1	78269	78351
	tmp_1_PossibleCrispr_2	Possible	23	1	NODE_1	78433	78494
	tmp_1_PossibleCrispr_3	Possible	26	1	NODE_1	87200	87309
	tmp_24_Crispr_1	Confirmed	36	21	NODE_24	20764	22309
	tmp_98_Crispr_1	Confirmed	32	7	NODE_98	18077	18549
	tmp_104_PossibleCrispr_1	Possible	39	1	NODE_104	9155	9259
	tmp_136_PossibleCrispr_1	Possible	31	1	NODE_136	4795	4887
L. fermentum QAULFN21	tmp_68_PossibleCrispr_1	Possible	39	2	NODE_68	11304	11483
	tmp_73_Crispr_1	Confirmed	36	12	NODE_73	49016	49843
	tmp_74_PossibleCrispr_1	Possible	33	1	NODE_74	219395	219500
L. fermentum QAULFN64	tmp_42_Crispr_1	Confirmed	36	6	NODE_42	20	451
L. fermentum QAULFN56	tmp_42_Crispr_1	Confirmed	36	6	NODE_42	20	451
L. delbrueckii QAULDN61	tmp_11_PossibleCrispr_1	Possible	46	1	NODE_10	113173	113173
	tmp_11_PossibleCrispr_2	Possible	27	1	NODE_10	120209	120319
	tmp_11_PossibleCrispr_3	Possible	52	1	NODE_10	461240	461240
	tmp_39_PossibleCrispr_1	Possible	23	1	NODE_17	53554	53554
	tmp_41_Crispr_2	Confirmed	28	42	NODE_42	67260	67260
	tmp_41_PossibleCrispr_1	Possible	8	1	NODE_42	1036	1162
L. delbrueckii QAULDN14	tmp_76_PossibleCrispr_1	Possible	23	1	NODE_76	2787	2878
	tmp_80_Crispr_1	Confirmed	36	10	NODE_80	642	1416
	tmp_88_PossibleCrispr_1	Possible	23	1	NODE_88	5255	5349
	tmp_123_Crispr_1	Confirmed	33	35	NODE_123	17944	20347
	tmp_130_Crispr_1	Confirmed	36	17	NODE_130	171	1464
L. acidophilus QAULAN51	tmp_20_Crispr_1	Confirmed	28	31	NODE_20	64554	64554
E. faecium QAUEFNS1	tmp_110_PossibleCrispr_1	Possible	25	1	NODE_107	3013	3121
	tmp_183_Crispr_1	Confirmed	24	3	NOE_207	2332	2531
E. faecium QAUEFNN4	tmp_1_PossibleCrispr_1	Possible	26	1	NODE_1	135508	135617
	tmp_2_PossibleCrispr_1	Possible	23	2	NODE_2	236591	236718
E. faecium QAUEFNA17	tmp_1_PossibleCrispr_1	Possible	26	1	NODE_1	324350	324459
	tmp_3_Crispr_1	Confirmed	24	3	NODE_3	11439	11638
	tmp_3_PossibleCrispr_2	Possible	33	1	NODE_3	46216	46328
E. faecium QAUEFNA13	tmp_14_Crispr_1	Confirmed	24	3	NODE_17	42535	42734
	tmp_39_PossibleCrispr_1	Possible	36	1	NODE_39	1741	1841

4.4 Discussion

Several possible benefits have been believed to be linked with the use of multi-strains probiotics, as compared with single-strain probiotics. Such as multi strains might have maximum chances of beneficial success and hence have a greater success rate for the desired health benefit. Multi strains offer a high diversity and thus have more possible functions and a broader range of effectiveness (Ouwehand *et al.*, 2018). Multi-strain probiotics should have preservative or synergistic effects: greater and strong attachment, formation of a good living environment, and declined antagonism of endogenous microbiota (Kwoji *et al.*, 2021). In this context, a multi strains probiotic community was developed from individual well characterized strains of probiotic bacteria isolated from traditionally fermented milk product Dahi. It is important to know whether the initial multi-strains mixture changed significantly during the experiments. But it is time taking and laborious to trace individual strains of the community. The community was characterized for growth, antagonistic effect against reference ATCC pathogens, *in vitro* cell surface hydrophobicity, auto-aggregation and cholesterol assimilation, the genomes of all the strains of the community were sequenced and analysed.

Based on two criteria such as diversity of the probiotics and survival of the probiotic strains in the simulated gastrointestinal fluids, two communities MSPC-1 (49 strains) and MSPC-2 (22 strains) were selected and evaluated. First, the communities were checked for synergism via testing their growth pattern as a community. The MSPC-1 had 1.21×10^5 cfu/ml while the MSPC-2 had 1.48×10^5 cfu/ml on Tryptic soya medium but the difference was not significant based on p value > 0.05. On skim milk the growth of MCPC-1 was 9.83×10^4 cfu/ml while the growth of MSPC-2 was 1.33×10^5 cfu/ml and the difference are significant (p value = 0.03). A similar study has been published where increased growth of bacteria recovered from canal roots and co-cultured as a community has been linked with synergism (Moreira *et al.*, 2011). Although in this way the individual growth of each strain cannot be determined, but still can be used an estimate for overall growth dynamics (Moreira *et al.*, 2011). The antagonistic effect of both the communities was evaluated against reference pathogens. It was observed that MSCP-2 has significant antagonist effect against *B. subtilis* and *S. pneumonia* i.e., 85% and 89% respectively (P. value < 0.05) when compared to MSPC-1's effect. This effect is equivalent to the effect that *L. fermentum*

QAULFN61 and *L. fermentum* QAULFN21 individually displayed against *B. subtilis* and *S. pneumoniae* that was the highest effect of all the individual strain's effect. Similarly, MSCP-1 had no antagonistic effect against *E. coli* and *S. enterica* while their growth was reduced up to 83% and 80% respectively by MSPC-2. This effect was also equivalent to the effect that *E. faecium* QAUEFNA13 and *L. fermentum* QAULFN21 individually displayed against *E. coli* and *S. enterica* that was the maximum effect against these pathogens. The antagonistic effect of MSPC-2 against *P. aeruginosa* and *S. aureus* is comparatively high but non-significant. This study is supported by the results of a recently published study in which a community of three lactic acid bacteria (*L. plantarum*, *L. acidophilus* and *L. casei*) has significantly reduced the growth of multi-drug resistant *Staphylococcus* strains (Bhola *et al.*, 2019). Another study also supports the finding of current study in which a cheese origin probiotic community was used against *L. monocytogenes* growth, that reduced its growth significantly and showed that reducing the diversity of probiotics in the community did not reduce the antagonistic effect (Imran *et al.*, 2010). The communities were evaluated for *in vitro* cell surface hydrophobicity and auto-aggregation. It was observed that both the communities displayed *in vitro* cell surface hydrophobicities of 97% and 98%, the difference is non-significant while the MSPC-2 had auto-aggregation of 83% that is significantly high (p – value = 0.03) from that of MSPC-1's auto-aggregation. Probiotics with high *in vitro* cell surface hydrophobicities are thought to last longer in the intestinal tract of the consumers (Krasowska *et al.*, 2014). Moreover, high auto-aggregation of probiotic has been linked with their antipathogenic and synergistic effect (Balakrishna & Technology, 2013). The microbial communities when were assessed for *in vitro* cholesterol assimilating abilities, it was observed that both the communities can assimilate 83% and 85% of the provided cholesterol in 24 hours of incubation. Numerous studies have been published on the individual cholesterol lowering characteristics of dairy origin bacteria, but there are no such studies on multi strains communities (Akbarzadeh & Homayouni, 2012). Based on these properties it can be concluded that the strains of MSPC-2 have synergistic effect for each other, can stay longer in the intestinal tract, and have more antipathogenic effect against reference pathogens.

The Small Intestine (TSI), *in vitro* model, which effectively simulates the actual intestinal conditions, was used to evaluate and estimate the survival of MSPC-2's strains in the actual

intestinal environment (Mahe *et al.*, 2017). Based on total cfu calculation, the growth of MSPC-2 reduced in the gastric phase from 1.36×10^4 cfu/ml to 4.55×10^3 cfu/ml during fasting state, and from 1.81×10^4 cfu/ml to 3.53×10^3 cfu/ml in feeding state. When observed the total OUT count, MSPC-2 OTUs reduced from 22027 to 886 during fasting state, and from 6591 to 2730 during feeding state. Reduction of probiotic growth in gastric phase can be linked with various factors such as low pH, pepsin activity, and mechanical churning (Martinsen *et al.*, 2005). Reduction of growth at genera level was also observed, when sample was collected after passing from the gastric phase. The number of *Lactobacillus* and *Enterococcus* was decreased from 20011 OTUs to 7339 OUTs and from 1537 OTUs to 448 OTUs respectively during fasting state. Their number was also decreased from 5766 OTUs to 2015 OTUs and from 402 to 290 OTUs during feeding state. Studies published on the viability of probiotics in gastric phase also show similar results where strains of *Bifidobacterium longum* and *Bifidobacterium breve* were undetectable at the end of gastric phase (Cook *et al.*, 2012). The number of *Streptococcus* and *Lactococcus* increased during gastric phase of fasting state from 72 to 165 OTUs and 408 to 875 OTUs respectively, the OTUs of *Lactococcus* also increased from 69 to 90 during gastric phase of feeding state. It has been reported that *Streptococcus* and *Lactococcus* modify its surface protein along with expression of some other specific genes that helps them to tolerate the harsh condition of stomach (Uriot *et al.*, 2021). Reduced growth of the MSPC-2 was observed at the end of the Jejunum, though during fating state total colonies of MSPC-2 were not decreased but decreased from 1.54×10^4 cfu/ml to 1.18×10^4 cfu/ml, the number of total OTUs reduced from 23013 to 11905 OTUs in fasting state and from 88280 to 53700 OTUs during feeding state. Reduction of probiotic growth in the Jejunum can be linked to nutrient depletion in the jejunum phase and to replenishing effect of the jejunum (Igam, 2019). Reduction in growth was also observed at genus level in both fasting and feeding state. It was observed that *Lactobacillus* decreased from 20690 OTUs to 10228 OTUs in fasting state from 80330 to 48952 OTUs in feeding state, *Streptococcus* decreased from 366 OTUs to 171 OTUs in fasting state while increased from 500 to 700 during feeding state. *Lactococcus* decreased 1545 OTUs to 651 OTUs in fasting state and from 325 OTUs to 116 OTUs during feeding state. *Enterococcus* increased from 411 OTUs to 854 OTUs during fasting phase and from 1125 OTUs to 3864 OTUs during feeding state. A similar

study has been recently published in which it has been reported that when the strains of *L. acidophilus* and *B. longum* passes through the jejunum their growth is reduced by 50% (Jacobsen *et al.*, 2021). Increased growth during feeding state can be linked with the buffering effect of the food (Matouskova *et al.*, 2021). After the gastric and jejunum phases, the isolates of lactic acid bacteria retained their growth, and at the end, they had the same number as they had before introduction to the model. Different mechanisms are associated with probiotic bacteria for their survival in harsh and stressed conditions of the gastrointestinal tract such as expression of genes in response to bile salts and enzymes (Han *et al.*, 2021), modification of surface protein in response to low pH (Uriot *et al.*, 2021), F_1F_0-ATPase proton pump reduction in response to low pH (Yao *et al.*, 2020), and activation of stress response mechanism (Papadimitriou *et al.*, 2016).

Genomes of all the 22 strains belonging to MSPC-2 were sequenced and analysed, the size of the genomes ranged between 1.8 to 3.1 Mb. Where the *L. delbrueckii* QAULDN61 contain smallest genome and *E. faecium* QAUEFNA17 contain largest genome in all the genomes presented in the study. Studies have reported the genome of lactic acid bacteria ranging from 1.2 Mb to 3.4 Mb (Davidson *et al.*, 1996). The GC contents of the genomes ranged from 34 to 52%, which is in line with published literature (Makarova *et al.*, 2006; O'sullivan *et al.*, 2009). When annotated with RAST tool, the genomes of lactic acid bacteria revealed minimum number of genes 1915 that were associated to the genome of *L. paracasae* QAULPN3 and maximum genes 3335 that were associated to the genome of *E. faecium* QAUEFNA17. A comprehensive review recently published on the genomes of lactic acid bacteria also states that the genome of lactic acid bacteria carries minimum genes of 900 to maximum of 4000 (Wu *et al.*, 2017). Based on SEED subsystem's data based the highest genes in the genomes are reserved for carbohydrate metabolism, protein metabolism, and amino acid metabolism. Among the reported genomes, highest number of genes responsible for carbohydrates metabolism are encoded by the genome of *L. rhamnosus* QAULRN2 while minimum genes coded for carbohydrate metabolism are associated with the genome of *L. delbrueckii* QAULDN64. Maximum genes for protein metabolism were coded by the genome of *E. faecium* QAUEFNA17A while minimum number of genes were coded by the genome of *L. delbrueckii* QAULDN64. Similarly, maximum number of genes for amino acid metabolism were encoded by the genome of *L.*

lactis QAULLNA8 and minimum were coded by the genome of *L. paracasae* QAULPN3. A recent study that checked carbohydrate metabolism genes in the genomes of lactic acid bacteria through phenotypic and genotypic method reported a high number of carbohydrate metabolism genes in the genome of lactic acid bacteria (Buron-Moles *et al.*, 2019). A recent large-scale study on the genomes of lactic acid bacteria has described the lactic acid bacteria genome as fragile and has linked its feature to specific niche and need of the bacteria (Pasolli *et al.*, 2020). And hence the protein and amino acid metabolism genes are the niche-specific (Bintsis, 2018). The EggNOG based COGs analysis also supports the presence of a major portion of genes responsible for metabolism and defence.

Though the lactic acid bacteria isolates, individually and in consortium showed antipathogenic activity against reference pathogenic strains, their genomes were also mined for the presence of bacteriocin genes. It was found that all the genomes had one or two bacteriocin genes in their genomes. In total, thirteen different bacteriocin genes were detected in the genomes of lactic acid bacteria. A recent study also reported 1 to 2 bacteriocin genes per genome of lactic acid bacteria (Collins *et al.*, 2017). The genomes were mined for the presence of antibiotic resistance genes, and it was found that 3 strains of *E. faecium* such as QAUEFNA17, QAUEFNN2 and QAUEFNS1 had a high number of antibiotic resistance genes. These strains were categorised as MDR due to showing resistance to more than three classes of antibiotics. However, these strains phenotypically had presented no multi-drug or extreme drug resistance. Similar studies have been published in recent years on the antibiotic resistance of *E. faecium* strains isolated from commercial probiotic products (Amachawadi, 2018; Wang, 2021). According to the study, most of the *E. faecium* strains show antibiotic resistance including MDR (Amachawadi, 2018). The antibiotic resistance *E. faecium* are also reported to have greater ability to stay and colonize in the intestinal tract (Amachawadi, 2018). The genomes were mined for the presence of virulence genes and Phage genes. It was found that *E. faecium* has no virulence and pathogenic genes but carries 7 to 11 Phage genes in their genomes. A recent study supports our finding where *E. faecium* isolated from different sources was mined for the presence of antibiotic resistance genes; they found many resistance genes in the genomes (Aun *et al.*, 2021). Furthermore, they assessed the transferability of the resistance genes and found that *E. faecium* did not spread antibiotic resistance genes (Aun *et al.*, 2021). The

E. faecium did not qualify for QPS or GRAS status, but due to potential probiotic characteristics, it has been used as probiotics after evaluating it safety at the genome and molecular basis (Baccouri *et al.*, 2019; Hanchi *et al.*, 2018b). The strains of *lactobacillus, Lactococcus,* and *Streptococcus* used in a current study carried no antibiotic resistance genes or virulence genes sticking to their GRAS status.

The genomes were evaluated for the presence of CRISPR genes, and it was found that all the genomes are CRISPRs in their genome. The presence of CRISPR confirms the immunity of these microorganisms to foreign attacking agents such as Phage, jumping genes, or transfer elements (Marraffini, 2015). From the current study, it can be concluded that the lactic acid bacteria strains used in the community are safe and can be used as potential probiotics.

4.5 Conclusion

The MSPC-2 presented high *in vitro* attachment and clump formation, survived well in the simulated environment of the small intestine. The MSPC-2 also showed significant *in vitro* antipathogenic activity that is confirmed through genome mining and by the presence of bacteriocin genes in their genomes. The genome analysis revealed putative functionality for repair and metabolism. Except, *E. faecium* strains no strain carried antibiotic resistance genes. The genomes had no pathogenic, virulence, or associated genes. The genome analysis reveals the adoption of immunity to foreign transfer elements

Chapter 5
Modulation of Obesity Related Gut Microbiota Dysbiosis with Multi Strains Probiotic Community

5.1 Introduction

In the last few decades, obesity has become a global health problem with an epidemic ratio affecting adults, teenagers, and children of both gender. The pathophysiology of obesity has been known to be very complex, so that many factors, such as behavioural factors, especially energy intake and physical activity, environmental factors, and genetic factors, are interactively involved in the onset of obesity (Paek *et al.*, 2018). The intestinal microbiota has appeared as a powerful, and vital endogenic feature that affects metabolism and energy homeostasis. Data supporting the association of human intestinal microbiota and obesity has expanded dramatically during the last few years, and the idea of gut microbiota dysbiosis mitigation has given a new insight into the cure and treatment of obesity (Requena *et al.*, 2021). Among many options, the utilization of probiotics for the modulation of human intestinal microbiota is a comparatively safe and non-invasive method. The most known probiotics are LAB strains belonging to the genera Bifidobacteria and Lactobacilli, which have a reputable safety record and have been given GRAS status by the FDA (Borriello *et al.*, 2003). In addition, bacterial probiotics that are still being searched include members of the genera *Bacillus, Enterococcus, Escherichia* and *Propionibacterium* (Grover *et al.*, 2012).

Numerous studies have stated that certain probiotic strains could decrease body weight and metabolic disorders, such as metabolic endotoxemia and insulin resistance. In human trials using stains of *L. rhamnosus* GG, *L. gasseri* BNR17*, L. gasseri* SBT2055*, B. breve* B-3*, L. casei* DN 114001, *B. lactis* BB-12, and *B. adolescentis* IVS-1 alone or in symbiotic form has proven their anti-obesity effects via specie and strain-specific mechanism in terms of satiety increase, bodyweight reduction or gut microbiota modulation (Barathikannan *et al.*, 2019; Wiciński *et al.*, 2020). Although experiments have been successful, it seems naturally impossible to modulate a community of million microbes using a single microbe. Hence, several studies have investigated the effects of multi-strain probiotics to determine their combinatorial efficacy compared to single-strain alternatives. For example, a combination of three probiotic strains (*S. thermophiles, L. bulgaricus, and B. lactis Bb*-12) resulted in weight loss in obese individuals after a ten weeks trial (Mohammadi-Sartang *et al.*, 2018). In the same way, preparation of nine probiotic strains, *B. bifidum* W23*, L. salivarius* W24*, L. acidophilus* W37*, B. lactis* W51*, B. lactis* W52*, L. casei* W56*, L. brevis*

W63*, Lactococcus lactis* W19, and *L. lactis* W58 administration in postmenopausal obese women for 12 weeks resulted in glucose, lipopolysaccharides, total cholesterol, and insulin reduction along with decrease in waist fringe and fats (Szulińska *et al.*, 2018). Similarly, a mixture of eleven strains comprising of *B. bifidum* SGB02*, B. animalis subsp. lactis* SGB06*, S. thermophilus* SGSt01*, L. plantarum* SGL07*, L. delbrueckii spp. bulgaricus* DSM 20081*, L. reuteri* SGL01, *L. acidophilus* SGL11*,* and *Lactococcus lactis subsp. lactis* SGLc01 was given orally to overweight and obese individuals for three weeks, resulting in significant body weight and subcutaneous fats reduction (Lorenzo *et al.*, 2017).

There is still speculation that more bacterial strains will have comparatively high potency for modulation of gut microbiota. That is why researchers are looking for a new multi-strain microbial combination that can significantly modulate the gut microbiota associated with obesity. In this context, current study aimed to investigate the effect of multi-strain probiotic community (MSPC-2) on obesity related gut microbiota dysbiosis and associated metabolism using a simulated *in vitro* CoMiniGut intestinal model..

5.2 Material and Methods

This study was conducted in the Department of Food Sciences, Section of Fermentation and Microbiology, University of Copenhagen, Denmark. The MSCP-2 (a multi-strains probiotic community), that was developed and characterized in Phase I and Phase II was used for evaluating its effect on obesity related gut microbiota. For this purpose, faecal samples collected from Pakistani origin obese volunteers were used and the experiment was performed in the *in vitro* CoMiniGut Model.

5.2.1 The *CoMiniGut*

The *CoMiniGut* model contains a container with five similar single-vessel, agitated, anaerobiotic reactors, which are pH supervised and managed. Each reactor comprises an attached crystal glass vial (FQ-2010, Fused quartz crucible, cylindrical, 10 ml, OD 22 mm × H 33 mm, 5 ml working volume; AdValue Technology, Tucson, AZ, USA) placed in a 150 ml polymethylmethacrylate (PMMA) booth. The anaerobiotic environment is attained via either combined gas in-outlets for blooming the sections with nitrogen (99.8%) to continue anaerobiosis. Resazurin wet indicators are utilized to sense the anaerobiosis (Anaerobe Indicator Test; Sigma-Aldrich, St. Louis, MO, USA). The PMMA section's

closure consists of a PMMA disk, and a changeable vacuum lubricated silicon rubber division (20420-U SUPELCO GR-2 Rubber Sheet Stock material; Sigma-Aldrich, St. Louis, MO, USA) carrying a pH probe inlet. The rubber sheet is permeable to the needle for pH management, sample collection, and providing the media if needed. The corresponding arrangement of the five reactor containers in one component is grounded on a magnetic agitator with five moving spots. The climate box is maintained at a temperature of 37°C by a mixing water bath coupled with a heat-circulating platter inside the climate box and an exterior temperature probe located in the box for response regulation. An airing scheme secures even temperature supply, and temperature cataloging (Temp 101A MadgeTech Temperature data logger) is achieved through trials. The pH is checked via a six-channeled pH meter and data logger (Consort multi-parameter analyzer C3040). The pH meter is linked to a computer running in-built MATLAB scripts for pH control (ver. R2015a; the MathWorks, Inc., Natick, MA, USA) that controls a multi-channeled syringe pump charged with syringes having 1 M NaOH. The syringes (10 ml; BD Biosciences, San Jose, CA, USA) are linked through tubes (VWR), and inoculation needles (Frisenette, Knebel, Østjylland, Denmark) hooked on the fermentation pots (Wiese *et al.*, 2018b).

5.2.2 Fecal inoculum and experimental set up

Fecal samples collected from three Pakistani healthy lean and three obese donors were separately homogenized in a 1:1 ratio with PBS/glycerol solution in a stomacher bag for 2×60s with the Stomacher (Stomacher 400; Seward, Worthing, UK) at standard speed and were stored at -80ºC for further processing. Participants providing consent, sampling, and using the fecal for *in vitro* fermentations have been approved by the Bioethical Committee (BEC) of Quaid-I-Azam University Islamabad, Pakistan, under protocol number BEC-FBS-QAU2018-109.

PBS-Glycerol preserved samples were thawed and further diluted with 0.1 M PBS pH ratio 1:1 on the day of the experiment. CoMiniGut reaction vessels containing 4.750 ml of media were then inoculated with 0.250 ml of fecal slurry alone and 4.50 ml BCM with 0.250 ml fecal slurry in addition to 0.250 ml of the probiotic community (MSPC-2) to the fermentation vessel, making a 5 ml of the final volume. All the samples were randomly duplicates.

5.2.3 *In vitro* fermentation media and conditions

Stimulated batch-culture fermentations were adjusted up and filled maintaining sterile conditions with 4.5ml basal colon medium BCM (0.5g/l bile salts, 2g/l peptone water, 2g/l yeast extract, 0.1g/l NaCl, 0.04g/l K_2HPO_4, 0.04g/l KH_2PO_4, 0.01g/l $MgSO_4 \times 7H_2O$, 0.01g/l $CaCl_2 \times 6H_2O$, 2g/l $NaHCO_3^-$, Hemin 0.002g/l, Vitamin K_1 10µl, Tween-80 2 ml, 0.5g/l L-Cysteine-HCl Sigma colon medium was chosen for screening purposes as also done previously (Tsitko *et al.*, 2019).

Two hours before the experiment, the water bath (60°C internal temperature) and ventilation were turned on, and the pH electrodes were calibrated. The magnetic bench's speed was adjusted at 250 rpm, and the fermentation was allowed to run for 24 hours. After completing the cycle, fermentation volume was collected in sterilized cryotubes and stored at -20ºC till further processing.

5.2.4 DNA Extraction

The Bead-Beat Micro AX Gravity Kit (A&A Biotechnology, Gdynia, Poland) was used for the DNA extraction from the fermentation product according to the manufacturer's protocol. Briefly, 200 µl of fermentation liquid was transferred to 1.5 ml sterilized microtube to which 450µl of BS suspension buffer was added. Subsequently, 20µl of lysozyme and 5µl of Mutanolysin were added. The sample was mixed well and incubated at 50ºC for 20 min. After 20 min of incubation, 900 µl of LSU lysis buffer and 20 µl proteinase K were added. Then the suspension was transferred into bead beat tubes having 1g of Zicronica/Silica 1 mm beads; the sample was transferred into a bead-beater and run for 60 sec at maximum power. The mixture was then incubated for 30 minutes at 50ºC, vortexing from time to time. After the incubation, the sample was centrifuged at 12000 rpm for 5 min, and 1 ml of the supernatant was loaded onto the assembled and pre-equilibrated (by loading 500µl of KIG solution) Micro AXD column and allowed to pass through the column through gravity. Then 600 µl of WIG first wash solution was added and allowed to pass through the column, and then the second wash solution W2 of 500 µl was added to the column. When the wash 2 solution was utterly passed through the column, 60 µl of E elution buffer was added onto the column and allowed to stand for 5 min. Finally, the extracted DNA was eluted into 1.5 ml sterilized microtubes having 5 µl of N

neutralizing buffer with 120 µl of Elution buffer. The concentration and purity of the extracted DNA were measured using Nano-Drop ND-1000 spectrophotometer (Saveen and Werner AB, Sweden).

5.2.5 16S rDNA amplicon sequencing

MinION (Oxford Nanopore Technologies, Oxford, UK) was used to sequence the 16S rDNA. Prior to library preparation, the extracted DNA was diluted to 10 ng/µL and the V1-V8 hypervariable region of 16S rRNA gene was amplified and sequenced with ONT using the following primers

ONT_27Fa: GTCTCGTGGG CTCGGAGATG TGTA TATAGA TCGCAGAGTT TGATYMTGGCTCAG, ONT_27Fb: GTCTCGTGGG CTCGGAGATG TGTA TATAGA TCGCAGAGTT TGATCCTGGCTTAG and ONT_1540_R: GTCTCGTGGG CTCGGAGATG TGTA TACTCT CTATTACGGY TACCTTGTTACGACT.

5.2.5.1 PCR-1 ON_UMI16S

The purified extracted DNA was diluted with sterilized Milli-Q® water to 1 ng/µl. PCR mix was prepared using PCRBIO Ultra Mix 12 µl, Sterilized Milli-Q® water 6 µl, Primer Mix 2 µl and 5 µl DNA for each sample. PCR was performed with 95ºC for 5 minutes. The 2 cycles of 95ºC for 20 seconds, 48ºC for 30 seconds, 65ºC for 10 seconds and 72ºC for 45 seconds followed by 72ºC for 4 minutes.

After completing PCR-1, the amplified DNA was visualized through 2% gel, and the quantity of DNA was measured through Quiblet. The PCR product was cleaned by transferring 18 µl of binding beads to 25 µl of PCR product and incubating at room temperature for 5 minutes. The product was washed twice with freshly prepared 80% ethanol.

5.2.5.2 PCR-2 ON_UMI16S

The 11 µl of the cleaned PCR-1 product was added to 12 µl of PCRBIO Ultra Mix and 2 µl of ONT barcodes (10 µM) into PCR tubes for second PCR. Furthermore, PCR 2 was run at 95ºC for 2 min, 33 cycles of 95ºC for 20 s, 55ºC for 20 s, and 72ºC for 40 s, with the final step of 72ºC for 4 min. The DNA obtained from PCR 2 was visualized with 2% gel and the quantity was measured with Quiblet.

The PCR 2 product was pooled for library preparation and was cleaned and then used for 16S sequencing through ONT.

5.2.6 Sequencing data analysis

Data produced by MinION were assembled using Min-Know software v19.06.8 (https://nanoporetech.com). The Guppy v3.2.2 basecalling toolkit was utilized to base call raw fast5 to fastq (https://nanoporetech.com). Porechop v0.2.2 was used for adapter trimming and sample demultiplexing (https://github.com/rrwick/Porechop). The Porechop adapter list was (adapters.py) edited accordingly. The quality scores of fastq files were corrected through NanoFilt ($q \geq 10$; read length >1Kb). Taxonomy assortment of quality corrected reads compared to GreenGenes (13.8) collection was performed by uclast method executed in parallel_assign_taxonomy_uclust.py (QIIME v1.9.1). The uclust settings were tuned on mock communities (−-similarity0.8; min_consensus_fraction 0.51) making sure annotations to the lowest possible taxonomic level with no false positive results. The settings permitted it to consider separate amplicon sequence options as separate "seeds" (Hui *et al.*, 2021). Reads categorized to at least phylum level was exposed for further analysis. The numbers of OTUs, taxonomy table, and mapping files were imported into the R tool, the microbiome package was used to analyze the samples.

5.2.7 Extraction and analysis of microbial metabolites

Metabolites associated with gut microbiota were extracted from the fermented slurry as explained previously (Wiese *et al.*, 2018a). Briefly, 1 ml slurry was taken from the total volume. The slurry was centrifuged at 8000 rpm for 5 minutes at ambient temperature for removal of the residual bacteria. After discarding the palette, supernatant was centrifuged at 14000 rpm for 15 minutes at 4ºC and was filtered through 0.45 µm filter. The filtrate was transferred into sterile microtube and used for further analysis. The filtrate was analysed using GC-MS (Agilent 7890A GC and an Agilent 5973 series MSD: Agilent, Waldbronn, Germany). GC separation was performed on a Phenomenex Zebron ZB-WAXplus column (30 m _ 250 mm _ 0.25 mm). A sample volume of 1 ml was injected into a split/split-less inlet at 285 _C using a 2:1 split ratio. Septum purge flow and split flow were set to 13 ml/min and 2 ml/min, respectively. Hydrogen was used as carrier gas, at a constant flow rate of 1.0 ml/min.

The GC oven program was as follows: initial temperature 100 _C, equilibration time 1.0 min, heat up to 120 _C at the rate of 10 _C/min, hold for 5 min, then heat at the rate of 40 _C/min until 230 _C and hold for 2 min. Mass spectra were recorded in Selected Ion Monitoring (SIM) mode and the following m/z ion were detected at a dwell time of 50 ms: 41, 43, 45, 57, 60, 73, 74, 84 and the MS detector was switched off during the 1 min of solvent delay time. The transfer line, ion source and quadrupole MS temperatures were set to 230, 230 and 150 _C, respectively. The mass spectrometer was tuned according to manufacturer's recommendation using perfluorotributylamine (PFTBA).

5.2.8 Statistical analysis

All the statistical analysis of data was performed with R (v3.6.2), SPSS and GraphPad Prism 9. The significant differences among groups were determined by ANOVA and Tukey's procedure for post hoc tests. For microbiome analysis R packages ggplot2, vegan, corrplot, Rhea, rstatix and vennDiagram were used.

5.3 Results

5.3.1 Effect of probiotics consortium on gut-bacterial count

The probiotic consortium affected the overall bacterial count and richness of the two experimental groups. It reduced the bacterial count in lean individuals and increased the number of bacteria in the obese group. The increase in richness was not significant. When seen at the individual level, it was observed that the probiotic treatment had promoted evenness in both groups.

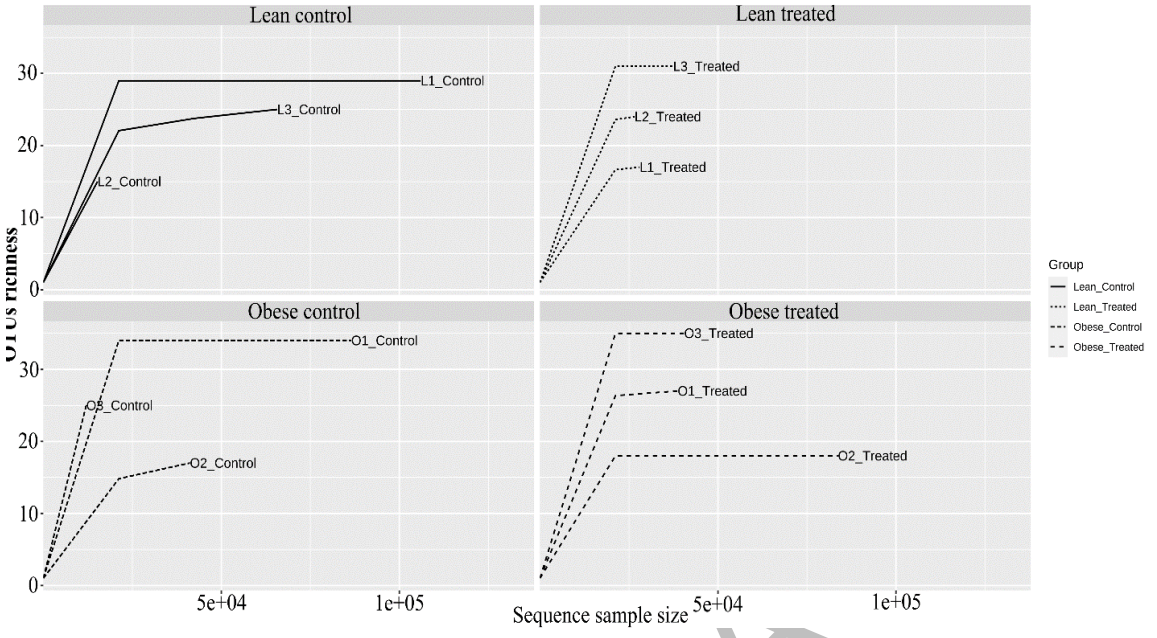

Figure 5. 1: Rarefaction curves comparing microbial diversity among treated groups after quality filtering.

5.3.2 Effect on bacterial diversity

5.3.2.1 Alpha Diversity

The probiotic microbial consortium had a positive effect on the gut microbiota diversity. Alpha diversity describes "within-sample" microbial diversity and measures how diverse a sample is in terms of microbiota. When assessed through Shannon's index, the alpha diversity was observed to be shifted from 0.9 to 0.8 (Mean) in the lean group while from 1.1 to 1.5 (Mean).

The alpha diversity assessed through Simpson's index also proved that the probiotic consortium could promote diversity in both groups. It shifted diversity from 0.7 to 0.8 in the lean group and from 0.6 to 0.7 in the obese group.

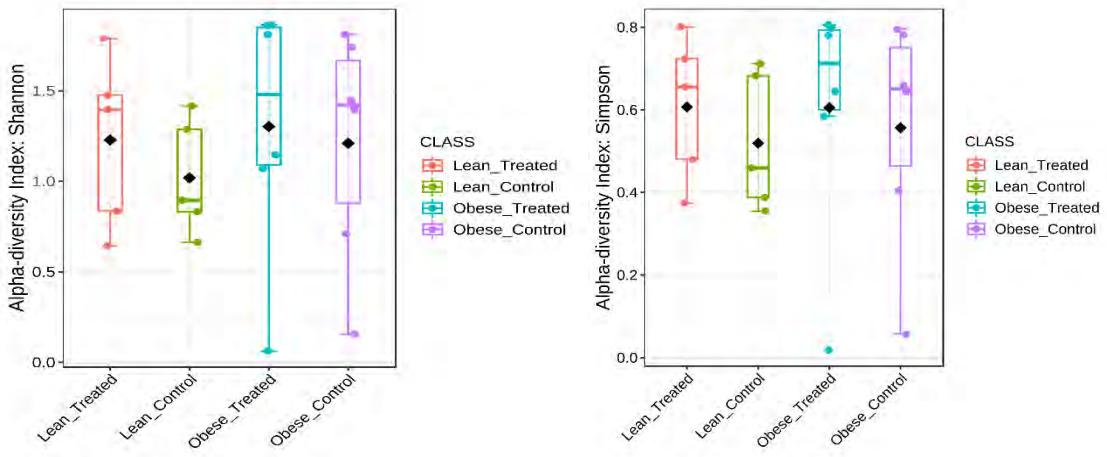

Figure 5. 2: Alpha diversity of lean and obese experimental groups assessed through Shannon and Simpson Indexes. Probiotic treatment increased alpha diversity in both lean and obese groups.

5.3.2.2 β-diversity

Beta diversity describes "between samples" microbial diversity. It measures similarity or dissimilarity of two samples in terms of microbiota. The probiotic microbial consortium was able to change the β diversity of the two experimental groups. It changed beta diversity in the lean group and obese group.

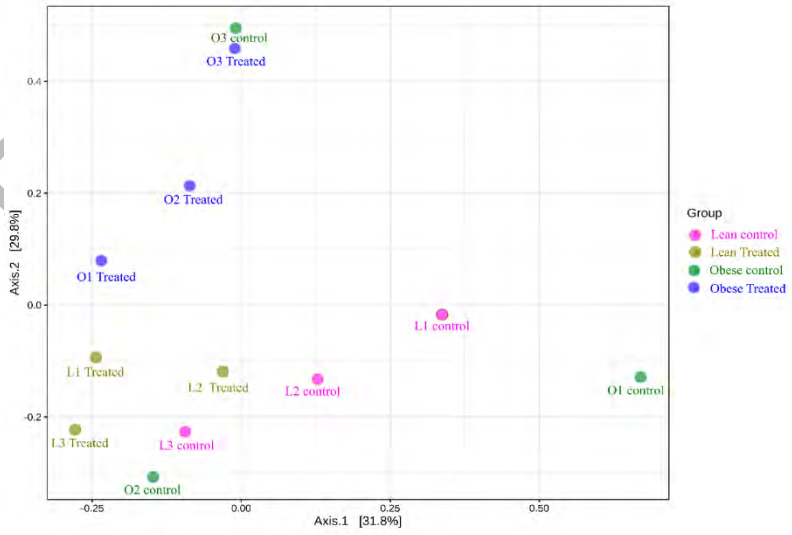

Figure 5. 3: Beta diversity analysis of microbial communities using principal component analysis based on OTUs based on Bray-Curtis. Probiotic treatment shifted beta diversity of the treated groups close to the control groups.

5.3.3 Effect of MSPC at the phylum level

The experimental groups' gut microbiota consisted of five phyla, namely *Actinobacteria*, *Bacteroidetes, Firmicutes, Proteobacteria,* and *Tenericutes.* Three phyla *Actinobacteria, Proteobacteria,* and *Firmicutes* were constituting 99% portion of the gut microbiota. The MSPC treatment changed the total and relative abundance of the three phyla. In the lean group the number of *Firmicutes* increased from 73% to 87%, the number of *Proteobacteria* decreased from 22% to 6%. In comparison, the number of *Actinobacteria* increased from 4% to 10%. In the obese group the number of *Actinobacteria* and *Proteobacteria* decreased from 16 to 4% and 32 to 6% respectively while the number of *Firmicutes* decreased from 89% to 51%. Detail results are depicted in Figure 5.4 and Figure 5.5

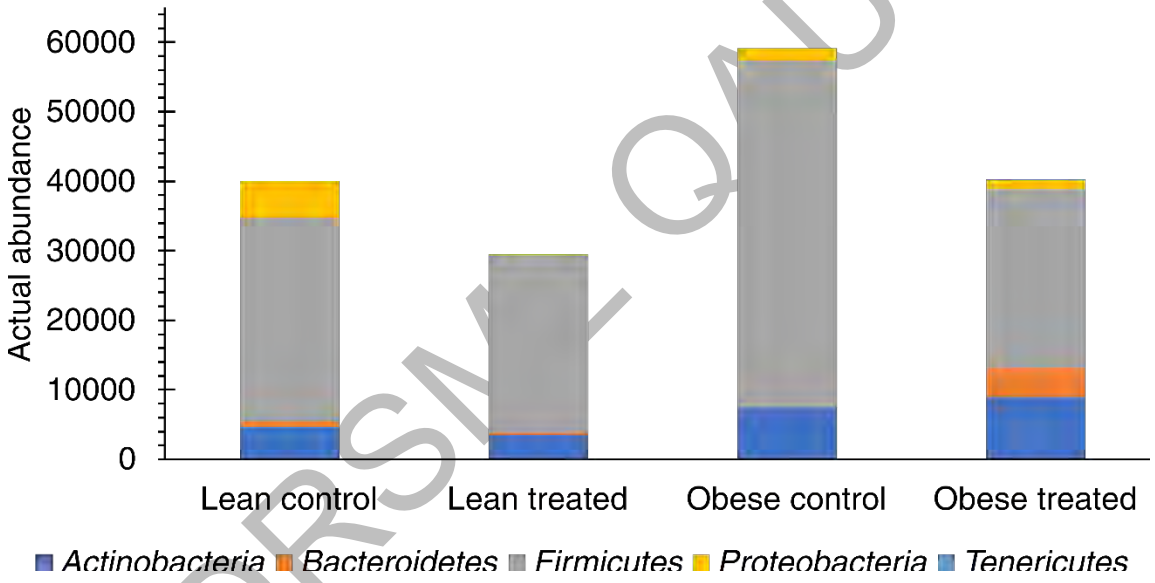

Figure 5. 4: Actual abundance of gut microbiota of obese and lean experimental group at the phylum level.

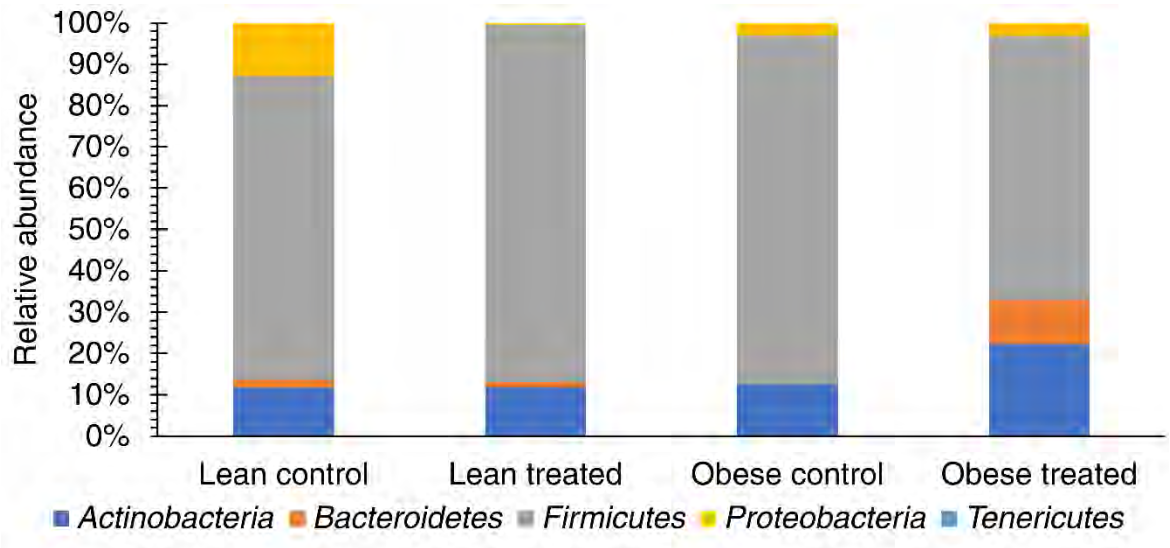

Figure 5. 5: Relative abundance of gut microbiota of lean and obese experimental groups at phylum level.

5.3.4 Effect of MSPC at genus level

Changes were also observed at the genus level as a result of MSPC treatment. The lean group before probiotic exposure had 24 genera, while after probiotic treatment, it contained 26 genera. The number of individual genera OTUs was also changed in response to probiotic treatment. The number of *Actinomyces* increased from 0.27 to 2.89%, *Anaerostipes* from 0.41 to 1.57%, *Clostridium* 16.83 to 30.54%, *Coprococcus* 0.13 to 1.37%, *Lactobacillus* 0.08 to 1.71% and *Roseburia* 0.01 to 1.45% in response to probiotic treatment. The number of *Bifidobacterium* decreased from 8.3 to 5.19%, *Dailister* 6.3 to 2.9%, *Enterococcus* 1.18 to 0.52%, and *Rummeliibacillus* from 63.4 to 49.21% in response to probiotic treatment. The detailed results are presented in Figure 5.6 and Figure 5.7. The genera that are significantly changed are presented in Figure 5.8. The number of genera was also changed in the obese group in response to probiotic treatment. Before treatment, the obese group contained 29 genera that changed to 27 in response to probiotic treatment. Some genera were increased due to probiotic treatment. The *Acetobacter* increased from 0.12% to 2.13%, *Bifidobacterium* increased from 3% to 16.3%, *Coprococcus* increased from 0.79 to 1.34%, *Lactobacillus* from 5.38% to 13.71% and *Prevotella* from 1.73 to 903%. Some genera were increased in response to MSPC treatment. The genera *Actinomyces* decreased from 7.34% to 2.23%, *Anaerostipes* decreased from 2.70% to 1.61%, *Clostridium* decreased from 9.68% to 7.47%, *Dailister* from 7.75% to 0.89%,

Enterobacter from 5.15% to 0.08%, *Escherichia* from 1.07% to 0.01% and *Sutterella* from 2.31% to 0.12%. Detailed results are displayed in Figure 5.6 and Figure 5.7. Genera that are significantly changed are presented in Figure 5.8.

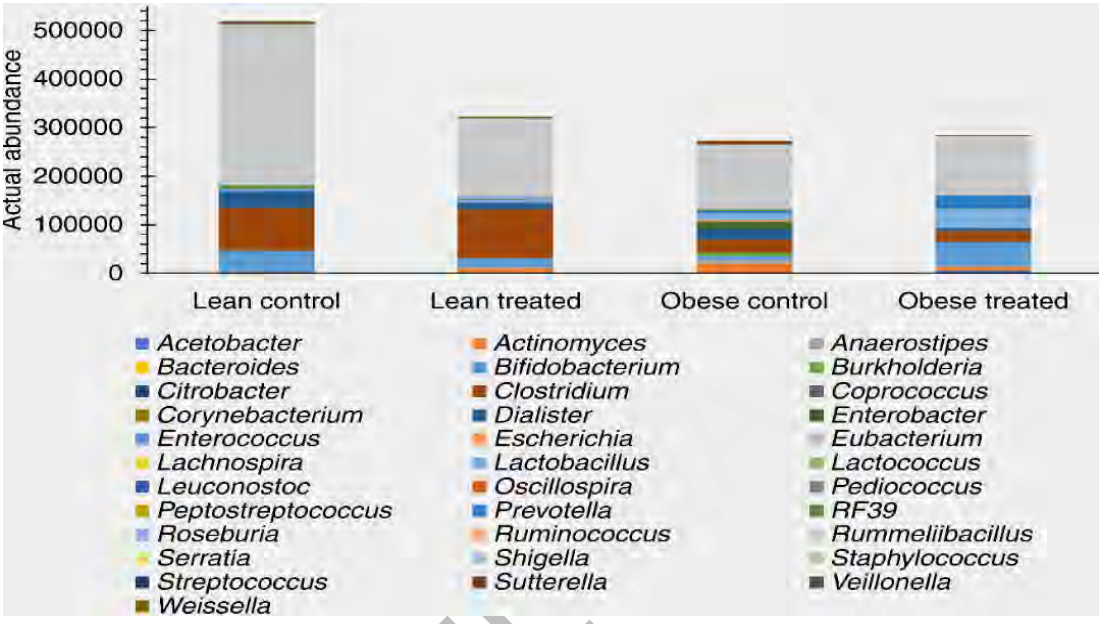

Figure 5. 6: Actual abundance of gut microbiota of lean and obese experimental group at genus level.

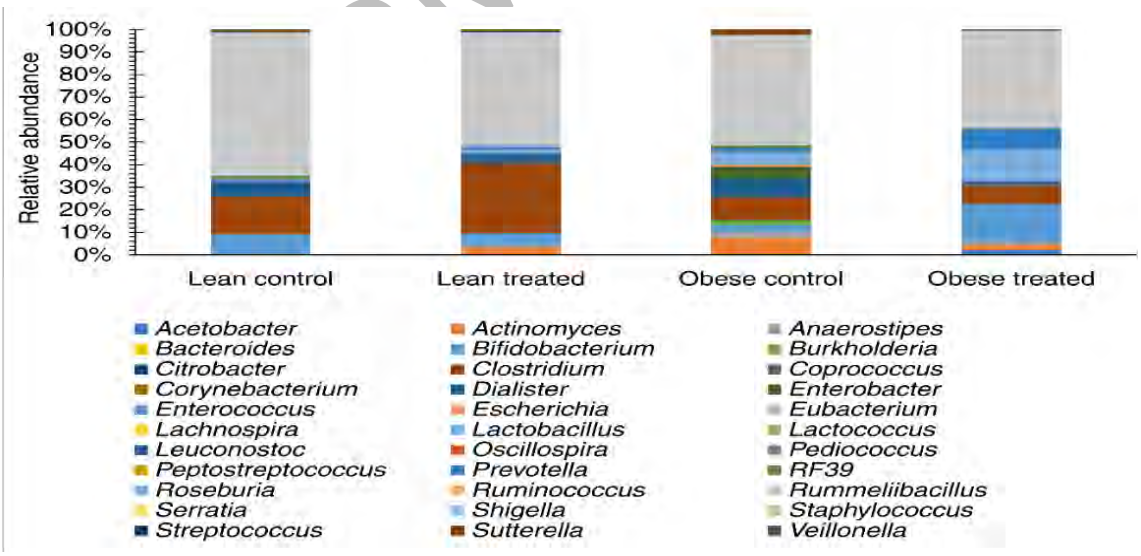

Figure 5. 7: Relative abundance of gut microbiota of lean and obese experimental groups at the genus level.

Bacterial Genera	Lean control	Lean treated	Obese control	Obese treated
Acetobacter	0	40	313	6058
Actinomyces	1414	9355	19972	6357
Bifidobacterium	43086	16764	8160	47361
Burkholderia	78	78	6285	285
Citrobacter	0	0	121	0
Coprococcus	717	4454	2162	3828
Dialister	33098	9619	21097	2531
Enterobacter	30	62	14011	222
Escherichia	27	25	2901	30
Lactobacillus	434	5534	14632	39055
Lactococcus	210	159	78	255
Pediococcus	28	0	646	8
Prevotella	230	2204	4621	25728
RF39	6852	298	2645	740
Roseburia	88	4698	55	1736
Rummeliibacillus	329006	158884	132101	121126
Shigella	1011	1158	252	78
Staphylococcus	106	0	835	499
Sutterella	3705	771	6289	332
Veillonella	114	165	107	607
Weissella	2543	2594	141	607

Increased
No change
Decreased

Figure 5. 8: Microbial genera that are significantly changed in both lean and obese experimental groups in response to probiotic treatment with a p-value =/≥ 0.05

5.3.4 Functional changes.

Predictive functional changes were also observed in response to probiotic treatment of both lean and obese groups.

5.3.4.1 Changes in COGs features

The COG-based features were changed in response to probiotic treatment. The genes responsible for carbohydrate transport and metabolism were increased in both lean and obese groups. In contrast, genes responsible for transcription and translation were increased in the lean group. The actual abundance and relative abundance of the COG are displayed in Figure 9 and Figure 10. Similarly, genes for cell wall synthesis, coenzyme transport and metabolism, inorganic transport and metabolism, intracellular trafficking secretion, and lipid transport and metabolism decreased in the lean group. Detailed results are displayed in Figure. 5.9 and Figure 5.10 The features that are significantly changed are presented in Table 5.1.

COGs in the obese group were also changed in response to probiotic treatment. The genes responsible for carbohydrate transport and metabolism, nucleotide transport and

metabolism, replication, recombination and repair, and translation, ribosomal structure, and biogenesis were increased in the obese group. Detailed results are presented in Figure 9 and 10. The COG features that are significantly changed are presented in Table 1. Similarly, the number of genes responsible for cell wall, membrane and envelope biogenesis, inorganic transport, and metabolism were decreased. Detailed results are presented in Figures 5.9 and 5.10. The features that are significantly changed are presented in Table 5.1.

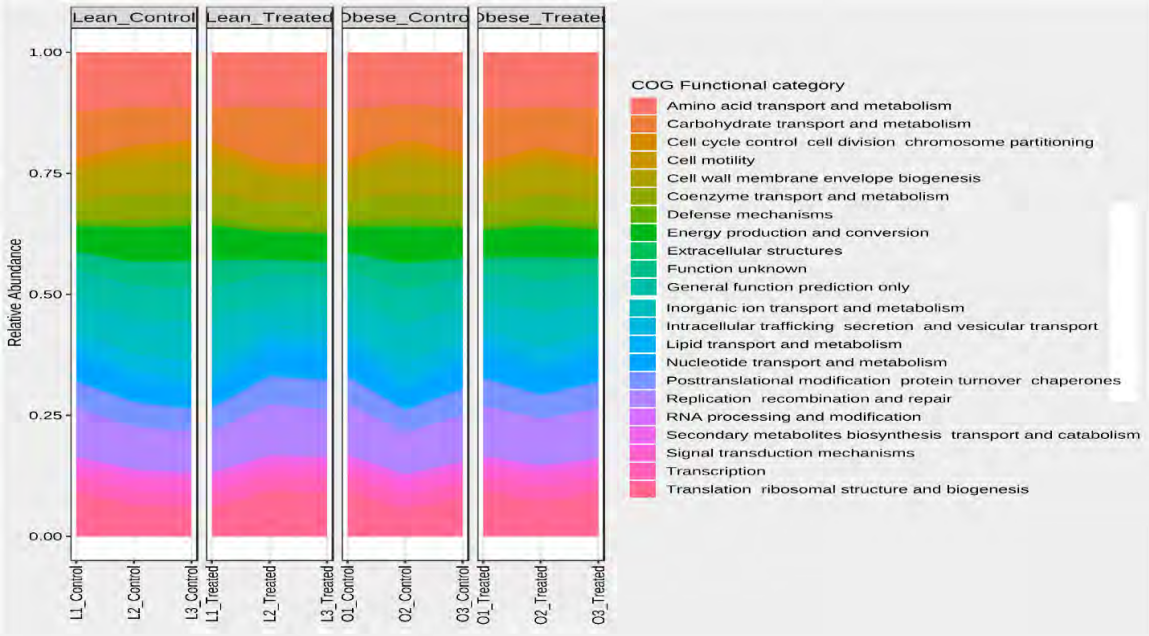

Figure 5. 9: Relative COG features of gut microbiota of lean and obese experimental groups.

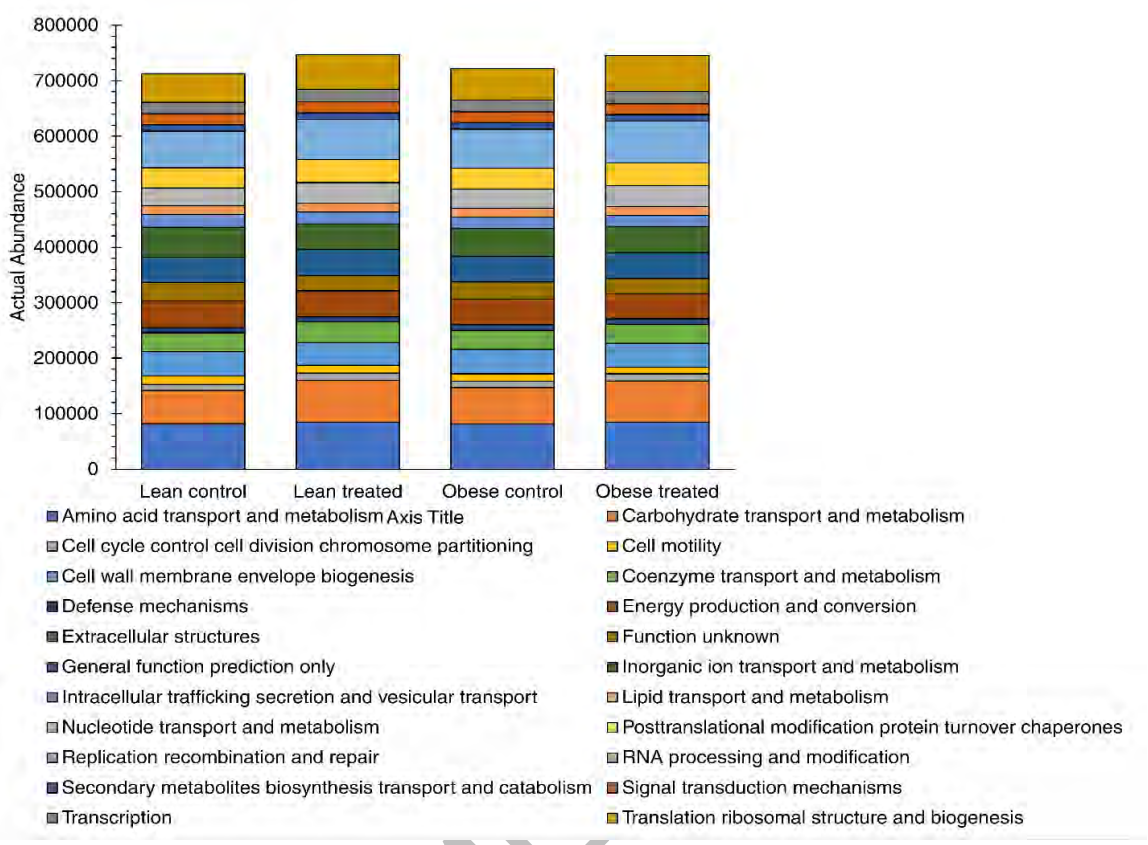

Figure 5. 10: Relative COG features of gut microbiota of lean and obese experimental groups.

Table 5. 1: COG features of lean and obese experimental group microbiota that are significantly changed in response to probiotic treatment.

Features	**Lean (*p-value*)**	**Obese (*p-value*)**
Cell wall membrane envelope biogenesis	0.002	0
Coenzyme transport and metabolism	0.001	0
Defense mechanisms	0.007	0.002
Energy production and conversion	0.001	0
Inorganic ion transport and metabolism	0.011	0.003
Intracellular trafficking secretion and Vesicular transport	0.01	0.002
Lipid transport and metabolism	0.002	0.002
Replication recombination and repair	0.01	0.002
Signal transduction mechanisms	0	0
Transcription	0.002	0.001

5.3.4.2 Changes in KEGG features

The MSPC treatment appeared to change KEGG predictive features. It was observed that the MSPC treatment increased the KEGG features in both the lean and obese experimental groups. The number of genes responsible for carbohydrate metabolism was not changed in the lean group but was increased in the obese group. Similarly, genes related to KEGG energy metabolism were increased in the lean group but not in the obese group. Detailed results are presented in Figure 5.11 and Figure 5.12. The p values are presented in Table 5.2.

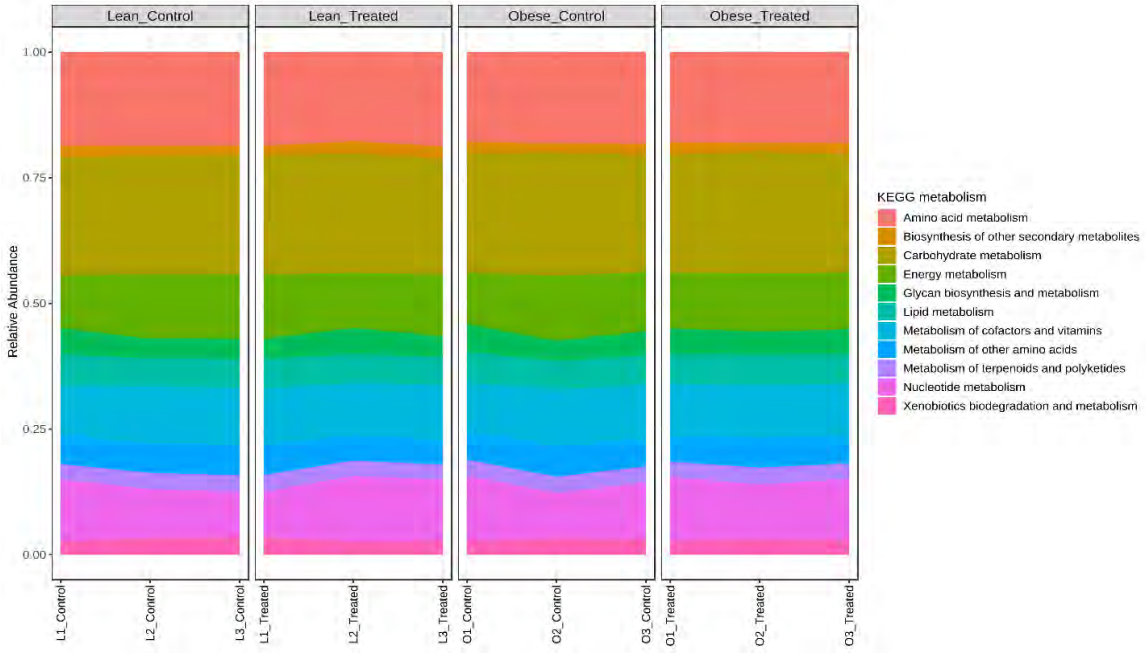

Figure 5. 11: Relative abundance of KEGG pathways of gut microbiota of lean and obese experimental groups.

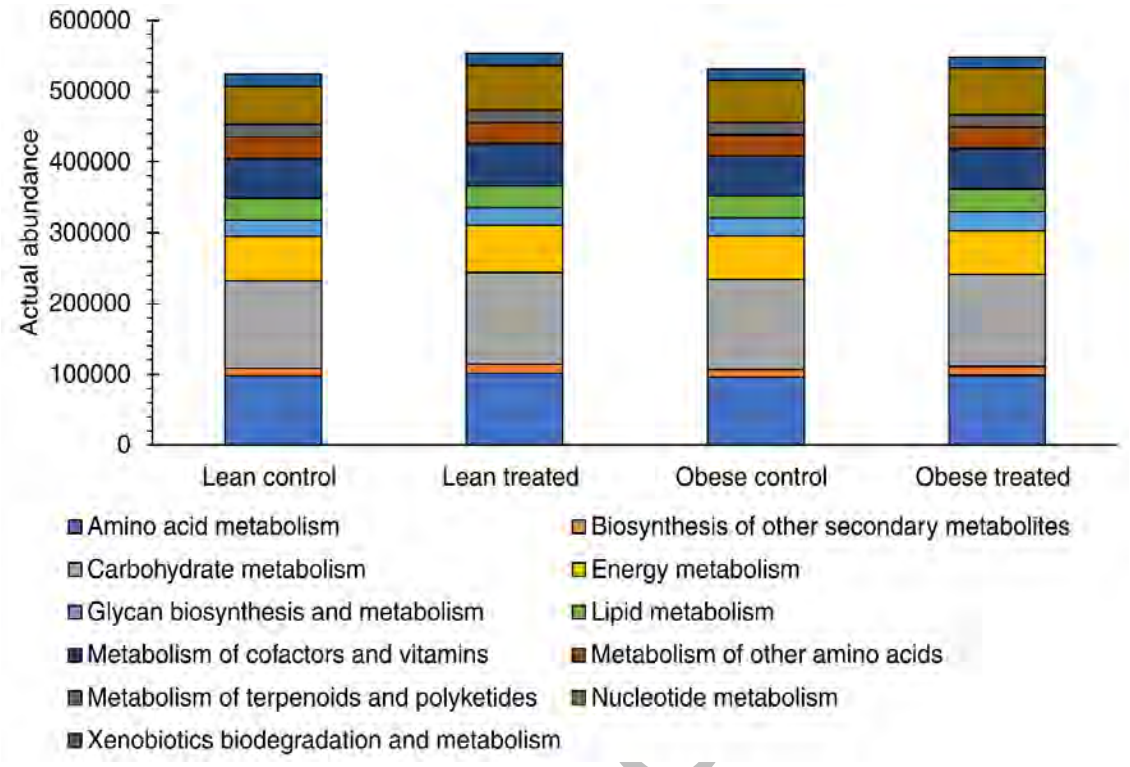

Figure 5. 12: Actual abundance of KEGG pathways of gut microbiota of lean and obese experimental groups.

Table 5. 2: KEGG pathways of Gut microbiota of lean and obese groups that are significantly changed in response to probiotic treatment.

Features	**Lean (p. values)**	**Obese (p. values)**
Amino acid metabolism	0.002	0.001
Carbohydrate metabolism	0.002	0
Energy metabolism	0.002	0
Lipid metabolism	0.001	0
Metabolism of cofactors and vitamins	0.002	0.001
Metabolism of other amino acids	0.001	0
Metabolism of terpenoids and polyketides	0	0
Nucleotide metabolism	0.02	0.02
Xenobiotics biodegradation and metabolism	0.002	0

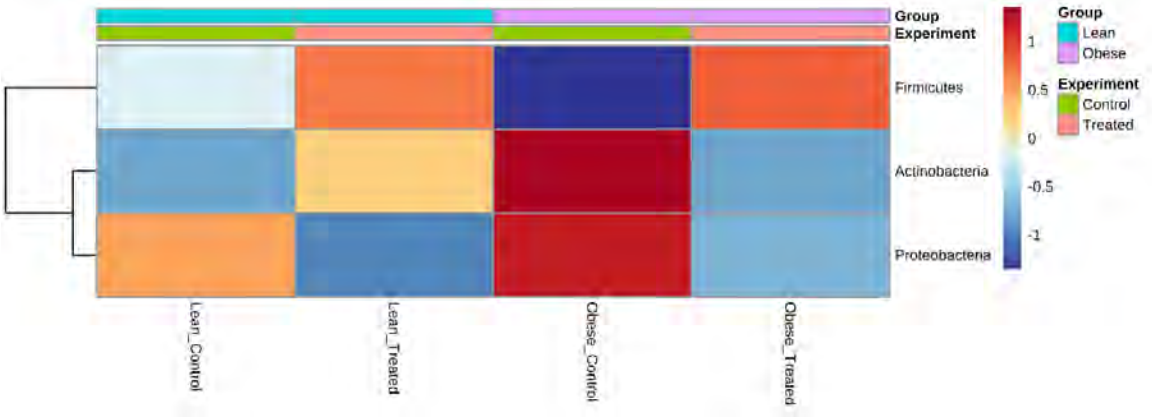

Figure 5. 13: Heatmap representing the gut microbiota of lean and obese groups at the phylum level

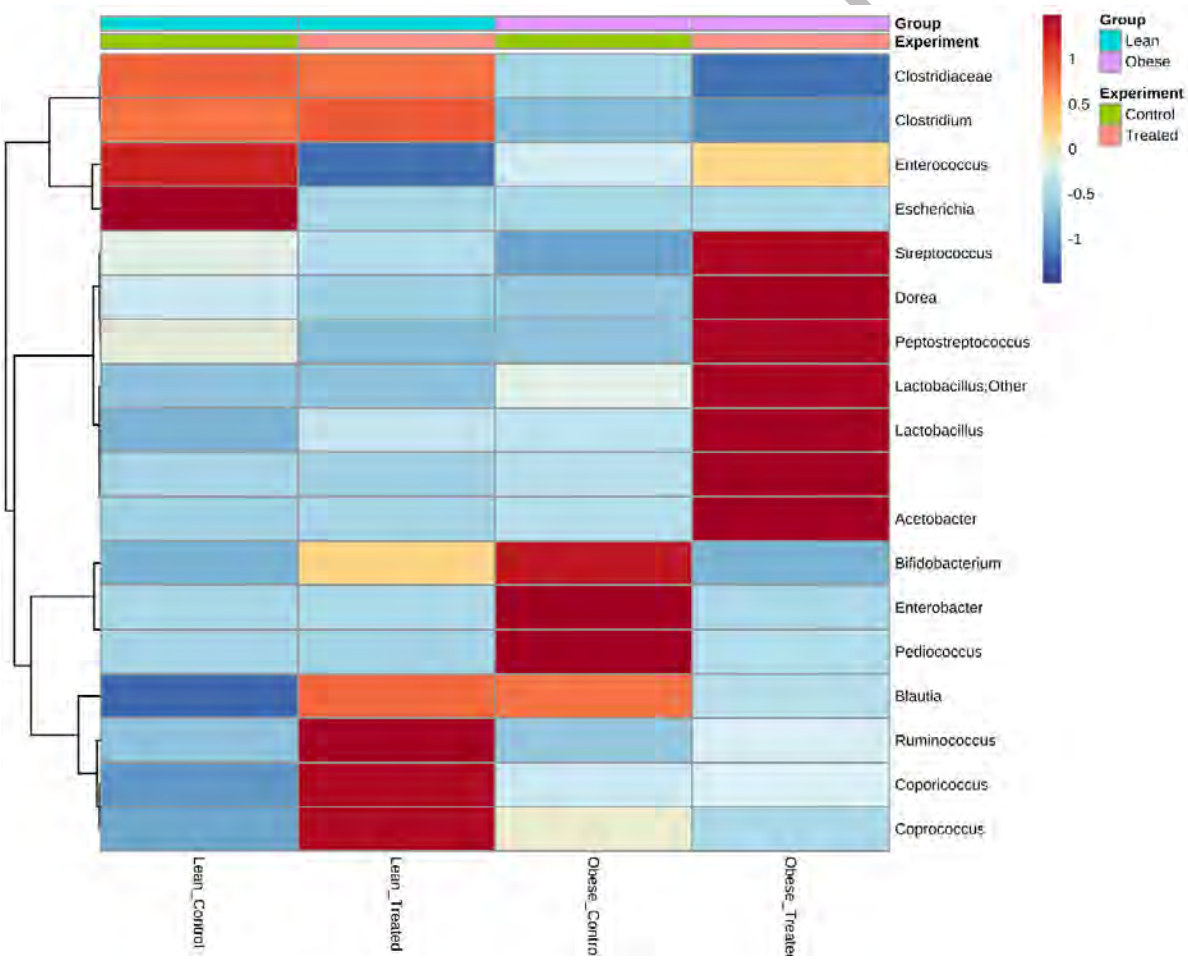

Figure 5. 14: Heatmap presenting the gut microbiota of lean and obese experimental groups at the genus level.

5.3.5 Changes in Gut Microbiota associated metabolites

The probiotic treatment also affected the type and concentration of fecal microbiota metabolites. Among selective metabolites, before probiotic treatment, in lean group, Diethyl Phthalate (3.26%), Valeric acid (5.2%), Butanoic acid (25.2%), N-methoxy-Methenamine (1.45%), and Propanoic acid (34.03%) were detected. While after probiotic treatment Butanoic acid (18.92%), Propanol (0.13%), Oleic acid (42.39) and Methyl 3-methoxyamino-propanoate (7.35%) were detected. Among these metabolites Butanoic acid was significantly ($p = 0.003$) affected due to probiotic treatment.

In the obese group, Butanoic acid was detected 9.22%, Acetic acid (18.42%), N-methoxy-Methanamine (3.83%) and Propionic acid (3.59%) were detected before probiotic treatment. After probiotic treatment Butanoic acid (10.24%), Methanamine, N-methoxy (27.8%) and Valeric acid (6.16%) were detected obese probiotic treated group. Results are presented in Figure 5.15.

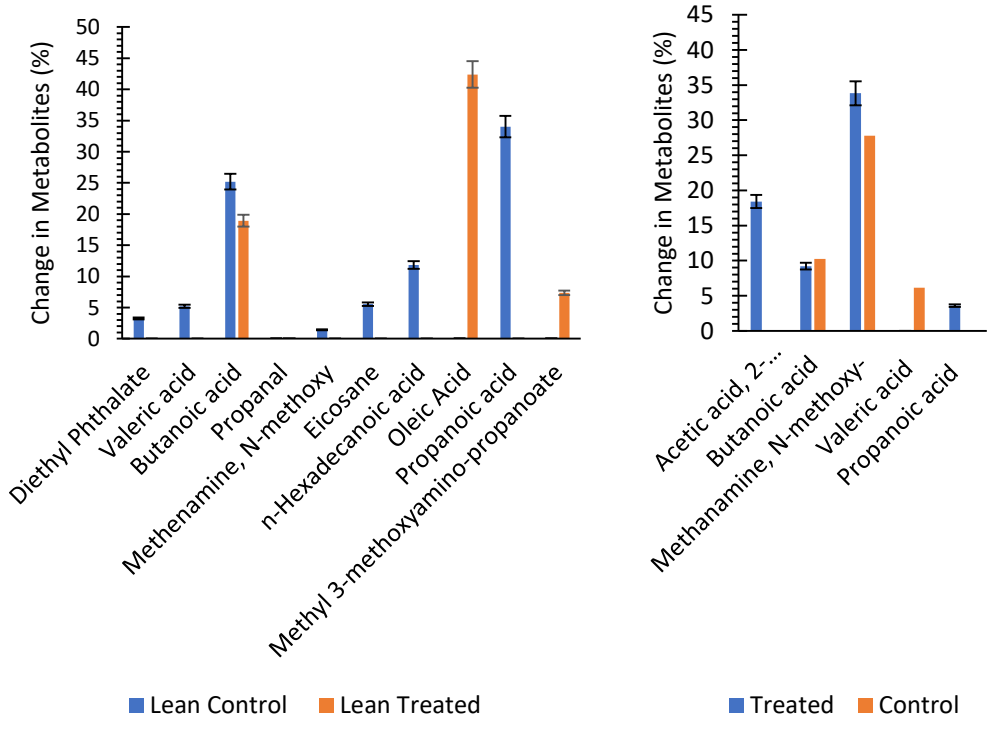

Figure 5. 15: Change in selective metabolites of Lean and Obese group's fecal microbiota in response to MSPC treatment. Statistically significant values ($p \leq 0.05$) are presented in bold fonts

5.4 Discussion

In the last few decades, obesity has become a global health problem with an epidemic ratio affecting adults, teenagers, and children of both sexes (Paek *et al.*, 2018). The intestinal microbiota has appeared as a potent and vital endogenic feature that affects metabolism and energy homeostasis, and the idea of gut microbiota mitigation has given a new insight into the cure and treatment of obesity (Requena *et al.*, 2021). Among many options, the utilization of probiotics for modulation of human intestinal microbiota is a comparatively safe and non-invasive method. It is speculated that multi-bacterial strains will have comparatively high potency for modulation of gut microbiota. In this context, we have developed a combination from pre characterized probiotics and have evaluated their effect on the gut microbiota associated with obesity using simulated CoMiniGut *in vitro* intestinal model.

Overall, increased count and reduced diversity in gut microbial communities are considered a sign of gut microbiota dysbiosis (Kriss *et al.*, 2018). It was observed that the MSPC treatment resulted in increased bacterial count in the obese group and promoted evenness in the gut microbial community, with an improvement in the Alpha and Beta diversity. The effect of probiotics on the obesity of gut microbiota was evaluated. Hence, the probiotics improved the α and β diversity of the obese subjects (Joseph *et al.*, 2020). It has been reported that at the phylum level, increased *Firmicutes/Bacteroidetes* are associated with obesity (Crovesy *et al.*, 2020). It was observed that the MSPC treatment increased the *Firmicutes* in the lean group and decreased in the obese group. Similar results are reported recently where administration of probiotic *Lactobacillus* is associated with a decrease in *Firmicutes* and *Bacteroidetes* in the faecal microbiota of obese subjects (Stojanov *et al.*, 2020).

At the genus level, increased pathogens and decreased beneficial microorganisms like *Lactococcus, Lactobacillus* and *Bifidobacterium* in the faecal microbiota are associated with dysbiosis and obesity (Hersoug *et al.*, 2018). In the present trial, we evaluated the effect of MSPC on the obesity-associated faecal microbiota. It was observed that the MSPC significantly increased the number of beneficial microorganisms especially *Lactobacillus, Lactococcus, Bifidobacterium,* and *Enterococcus,* in the faecal microbiota taken from obese subjects. The MSPC significantly decreased pathogens like *Escherichia,*

Enterobacter, Shigella, and *Staphylococcus* in the obesity-associated faecal microbiota. Studies have reported the increased number of *Bifidobacteria* after its administration in the faecal microbiota of the human obese subjects (Sanchis-Chordà *et al.*, 2019). The *Bifidobacteria* spp decreased the number of pathogen and improved the biomarkers responsible for inflammation (Sanchis-Chordà *et al.*, 2019). Similarly, *L. paracasae* was also able to reduce the number of Gram negatives in the faecal microbiota of obese subjects and decrease the inflammatory cytokines (Karlsson Videhult *et al.*, 2015). Similar results were reported for the strains of *L. rhamnosus* and *L. casei* (Callaway *et al.*, 2019). The effect of multi-species and strains of probiotics with different dosages on the obesity and associated gut microbiota was evaluated, and it was found that the multi-species and strains are more effective in terms of improvement in obesity biomarkers and obesity gut microbiota than single strains, moreover, probiotic effect is also dosage-dependent (Szulińska *et al.*, 2018).

COGs and KEGGs features associated with the gut microbiota were also predicted. It was found that the COGs and KEGGs features responsible for energy metabolism, food uptake, metabolism, defensive mechanisms, replication, recombination, and repair were improved in obese subjects in response to the MSPC treatment. Changes in the KEGGs and COGs associated with gut microbiota in response to probiotics consumption are currently reported. Where features responsible for defensive mechanisms, metabolism and reproduction were increased (Eloe-Fadrosh *et al.*, 2015).

The probiotic treatment also affected the type and concentration of microbiota metabolites. Among selective metabolites, Diethyl Phthalate (3.26%), Valeric acid (5.2%) and N-methoxy-Methenamine (1.45%), Eicosane (5.56%), n-Hexadecanoic acid (11.83%) and Propanoic acid (34.03) were effected in both the groups. The metabolite Diethyl Phthalate cannot be characterized as a potential harmful substance for human body but sometimes it may act as a toxic substance and can effect human health (Weaver *et al.*, 2020). Similarly, Valeric acid is known as a ligand for G-protein couple receptor which influence metabolism, immunity, and blood pressure (Le *et al.*, 2003). But an increase in the concentration of valeric has been associated with inflammatory markers such as C reactive protein and white blood count (Yamashiro *et al.*, 2017). The N-Methoxy-N-methylacetamide is believed that it has anti-inflammatory and anti-cancerous activity

(Musini et al., 2015). The metabolite Eicosane has detected in the body odor of persons suffering from Parkinson's disease (Trivedi *et al.*, 2019). Whereas inflammation and Parkinson disease have strong correlation with each other (Mcgeer & Mcgeer, 2004). Moreover, N=Hexadecanoic acid is believed to have anti-inflammatory properties (Aparna *et al.*, 2012). Propanoic acid acts as PPAR agonists that act upon the peroxisome proliferator-activated receptor and cures the symptoms of the metabolic syndrome, mainly for lowering triglycerides and blood sugar (Balint & Nagy, 2006). The Methyl 3-methoxyamino-propanoate is believed to be a skin allergen (Angelini *et al.*, 1995). After probiotic treatment, Butanoic acid and Oleic acid were detected in lean group. Butanoic acid has a role in the inhibition of chronic inflammation, strengthen gut barrier function, and perform immune regulatory functions (Sanz *et al*., 2015). Oleic acid has a role in prevention of cancer, inflammation, and autoimmune diseases (Sales-Campos et al., 2013). It also acts as prebiotic for the probiotics and enhances their survival in the intestinal tract of their hosts (Corcoran et al., 2007).

The MSPC increased microbial diversity and promoted homogeneity in the dysbiosis faecal microbiota. MSPC decreased *Firmicutes* in the obese samples. Increased the number of beneficial bacteria like *Lactococcus, lactobacillus,* and *Bifidobacterium and d*ecreased the pathogens like *Enterobacter*, *Escherichia,* and *Shigella*. MSPC increased metabolites that can treat obesity.

Based on current findings, it can be concluded that the MSPC can be used for modulation of the obesity-associated dysbiosis gut microbiota in the *in-vivo/*animal trial experiments.

5.5 Conclusion

The MSPC increased microbial diversity and promoted homogeneity in the dysbiosis gut microbiota. MSPC decreased *Firmicutes* significantly in the obese samples. Increased the number of beneficial bacteria like *Lactococcus, lactobacillus,* and *Bifidobacterium and d*ecreased the pathogens like *Enterobacter*, *Escherichia,* and *Shigella*. Based on functionality, increased COGs for nutrient uptake, metabolism, defence, cell regeneration, and repair. KEGG for metabolism is increased

Study Conclusions

Study conclusions

The isolates that were lactose utilizers, acid producers, and curd formers, belonged to lactic acid bacteria when their identity was confirmed through Rep-seq technique.. The bacteria presented desirable probiotic traits such as survival in low pH (2), high bile salts (0.35%), and high in vitro cell surface hydrophobicity and auto-aggregation (>80%), they were unable to tolerate in vitro simulated gastric fluids. The antagonistic activity toward different pathogens is strain dependent feature. The i*n vitro* safety assessment confirms the GRAS status of the lactic acid bacteria isolated from fermented milk product Dahi.

The MSPC-2 presented high *in vitro* attachment and clump formation, survived well in the simulated environment of small intestine. The MSPC-2 showed significant antipathogenic activity against reference pathogens. The genomes of MSPC-2 strains revealed putative functionality for repair and metabolism. Except, the strains of *E. faecium,* no antibiotic resistance genes were found in the genomes of the tested strains. The genomes had no pathogenic, virulence, or associated genes and were also found having adopted immunity to foreign transfer elements.

Treating gut microbiota dysbiosis with MSPC-2 resulted in an increased microbial diversity and promoted homogeneity in the gut microbiota. It decreased *Firmicutes* significantly in the obese samples and increased beneficial bacteria such as., *Lactococcus, lactobacillus* and *Bifidobacterium.* The MSPC-2 treatment decreased pathogens such as., *Enterobacter*, *Escherichia* and *Shigella* in both the tested groups. Based on functionality, it increased COGs for nutrient uptake, metabolism, defence, cell regeneration and repair in both the groups. KEGG for metabolism is also increased in both the experimental groups.

Prospects for the future

Prospects for the future

- Probiotics yeasts and Bacteriophages may be isolated, identified, and characterize for beneficial modulation of human gut microbiota.
- Antitoxic activity of the selected LAB isolates can be assessed.
- Cytotoxicity and *in-vivo* safety experiments can be performed for the selected LAB isolates.
- Different combinations of the LAB can be used to define better functional multi strains microbial consortia, such as excluding *Enterococcus* strains or using only *L. fermentum* isolates.
- Animal models can be used for the Gut modulation trails.
- To better understand the effect of MSPC, the duration of the experimental trial can be extended, and a whole metagenomic analysis can be performed for Gut Microbiota.

References

Abarca-Gómez, Leandra, Abdeen, Ziad A, Hamid, Zargar Abdul, Abu-Rmeileh, Niveen M, Acosta-Cazares, Benjamin, Acuin, Cecilia, Adams, Robert J, Aekplakorn, Wichai, Afsana, Kaosar, & Aguilar-Salinas, Carlos A. (2017). Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128· 9 million children, adolescents, and adults. *The Lancet, 390*(10113), 2627-2642.

Abushelaibi, Aisha, Al-Mahadin, Suheir, El-Tarabily, Khaled, Shah, Nagendra P, & Ayyash, Mutamed. (2017). Characterization of potential probiotic lactic acid bacteria isolated from camel milk. *LWT-Food science and Technology, 79*, 316-325.

Adam, Tanja C, Epel, Elissa S (2007). Stress, eating and the reward system. *Journal of Physiology, & behavior. 91*(4), 449-458.

Addi, N, & Guessas, B. (2015). Characterization of protease activity of *Lactococcus lactis* species isolated from raw camel's milk. *Journal of Biological Sciences, 16*(6), 215-220.

EFSA, Panel on Additives, Products or Substances used in Animal Feed (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA journal 10*(6), 2740.

Adjei-Fremah, Sarah, Ekwemalor, Kingsley, Worku, Mulumebet, & Ibrahim, Salam. (2018). Probiotics and ruminant health. *Probiotics: Current Knowledge and Future Prospects*, 133.

Adriaanse, Marieke A, de Ridder, Denise TD, Evers, Catharine (2011). Emotional eating: Eating when emotional or emotional about eating?*, Journal of Psychology, & Health*. *26*(1), 23-39.

Akbarzadeh, Fariborz, & Homayouni, Aziz. InTech. (2012). Dairy probiotic foods and coronary heart disease: A review on mechanism of action. *Probiotics*. 121-128.

Al-Attas, Omar S, Al-Daghri, Nasser M, Al-Rubeaan, Khalid, da Silva, Nancy F, Sabico, Shaun L, Kumar, Sudhesh, McTernan, Philip G, & Harte, Alison L. (2009). Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. *Cardiovascular diabetology, 8*(1), 20.

Albano, C, Morandi, S, Silvetti, T, Casiraghi, MC, Manini, F, & Brasca, M (2018). Lactic acid bacteria with cholesterol-lowering properties for dairy applications: In vitro and in situ activity. *Journal of dairy science. 101*(12), 10807-10818.

Alcock, Brian P, Raphenya, Amogelang R, Lau, Tammy TY, Tsang, Kara K, Bouchard, Mégane, Edalatmand, Arman, Huynh, William, Nguyen, Anna-Lisa V, Cheng, Annie A, & Liu, Sihan. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic acids research. 48*(D1), D517-D525.

Allen, Jacob M, Mailing, Lucy J, Niemiro, Grace M, Moore, Rachel, Cook, Marc D, White, Bryan A, Holscher, Hannah D, & Woods, Jeffrey A. (2018). Exercise alters gut microbiota composition and function in lean and obese humans. *Medicine & Science in Sports & Exercise, 50*(4), 747-757.

Álvarez-Cisneros, Yenizey M, & Ponce-Alquicira, Edith. (2018). Antibiotic Resistance in Lactic Acid Bacteria. In *Antimicrobial Resistance-A Global Threat*: IntechOpen.

Amar, Jacques, Burcelin, Rémy, Ruidavets, Jean Bernard, Cani, Patrice D, Fauvel, Josette, Alessi, Marie Christine, Chamontin, Bernard, & Ferriéres, Jean. (2008). Energy intake is associated with endotoxemia in apparently healthy men. *The American journal of clinical nutrition, 87*(5), 1219-1223.

Anal, Anil Kumar (2019). Quality ingredients and safety concerns for traditional fermented foods and beverages from Asia: A review. *Fermentation 5*(1), 8.

Angmo, Kunzes, Kumari, Anila, & Bhalla, Tek Chand. (2016). Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT-Food science and Technology, 66*, 428-435.

Aoun, Antoine, Darwish, Fatima, Hamod, Natacha (2020). The influence of the gut microbiome on obesity in adults and the role of probiotics, prebiotics, and synbiotics for weight loss. *Preventive Nutrition, & Science, Food*. *25*(2), 113.

Arndt, David, Grant, Jason R, Marcu, Ana, Sajed, Tanvir, Pon, Allison, Liang, Yongjie, & Wishart, David S (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic acids research*. *44*(W1), W16-W21.

Assad-Bustillos, Melissa, Palier, Juliette, Rabesona, Hanitra, Choiset, Yvan, Della Valle, Guy, & Feron, Gilles (2020). Role of the bolus degree of structure on the protein digestibility during in vitro digestion of a pea protein-fortified sponge cake chewed by elderly. *Journal of texture studies 51*(1), 134-143.

Attri, Sampan, & Goel, Gunjan (2018). Influence of polyphenol rich seabuckthorn berries juice on release of polyphenols and colonic microbiota on exposure to simulated human digestion model. *Food Research International. 111*, 314-323.

Aun, Erki, Kisand, Veljo, Laht, Mailis, Telling, Kaidi, Kalmus, Piret, Väli, Ülo, Brauer, Age, Remm, Maido, & Tenson, Tanel (2021). Molecular Characterization of *Enterococcus* Isolates From Different Sources in Estonia Reveals Potential Transmission of Resistance Genes Among Different Reservoirs. *Frontiers in microbiology 12*, 585.

Aw, Wanping, & Fukuda, Shinji (2019). Protective effects of bifidobacteria against enteropathogens. *Microbial biotechnology*. *12*(6), 1097-1100.

Awoyemi, Ayodeji, Trøseid, Marius, Arnesen, Harald, Solheim, Svein, Seljeflot, Ingebjørg (2018). Markers of metabolic endotoxemia as related to metabolic syndrome in an elderly male population at high cardiovascular risk: a cross-sectional study. *Diabetology, & Syndrome, Metabolic 10*(1), 59.

Babcock, Alicia A, Wirenfeldt, Martin, Holm, Thomas, Nielsen, Helle H, Dissing-Olesen, Lasse, Toft-Hansen, Henrik, Millward, Jason M, Landmann, Regine, Rivest, Serge, & Finsen, Bente. (2006). Toll-like receptor 2 signaling in response to brain injury: an innate bridge to neuroinflammation. *Journal of Neuroscience, 26*(49), 12826-12837.

Baccouri, Olfa, Boukerb, Amine Mohamed, Farhat, Leila Ben, Zébré, Arthur, Zimmermann, Kurt, Domann, Eugen, Cambronel, Mélyssa, Barreau, Magalie, Maillot, Olivier, & Rincé, Isabelle (2019). Probiotic potential and safety evaluation of Enterococcus faecalis OB14 and OB15, isolated from traditional tunisian testouri cheese and rigouta, using physiological and genomic analysis. *Frontiers in microbiology 10*, 881.

Bäckhed, Fredrik, Ding, Hao, Wang, Ting, Hooper, Lora V, Koh, Gou Young, Nagy, Andras, Semenkovich, Clay F, & Gordon, Jeffrey (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the national academy of sciences 101*(44), 15718-15723.

Bakhti, Mostafa, Böttcher, Anika, & Lickert, Heiko (2019). Modelling the endocrine pancreas in health and disease. *Nature Reviews Endocrinology 15*(3), 155-171.

Balakrishna, Aparna (2013). *In vitro* evaluation of adhesion and aggregation abilities of four potential probiotic strains isolated from guppy (Poecilia reticulata). *Brazilian Archives of Biology, & Technology*. *56*, 793-800.

Balint, B. L., & Nagy, L. (2006). Selective modulators of PPAR activity as new therapeutic tools in metabolic diseases. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 6*(1), 33-43

Bamola, V Deepak, Ghosh, Arnab, Kapardar, Raj Kishor, Lal, Banwari, Cheema, Simrita, Sarma, Priyangshu, Chaudhry, Rama (2017). Gut microbial diversity in health and disease: experience of healthy Indian subjects, and colon carcinoma and inflammatory bowel disease patients. *Microbial ecology in health, & disease*. *28*(1), 1322447.

Bankenahally, R, & Krovvidi, H (2016). Autonomic nervous system: anatomy, physiology, and relevance in anaesthesia and critical care medicine. *Bja Education 16*(11), 381-387.

Barathikannan, Kaliyan, Chelliah, Ramachandran, Rubab, Momna, Daliri, Eric Banan-Mwine, Elahi, Fazle, Kim, Dong-Hwan, Agastian, Paul, Oh, Seong-Yoon, & Oh, Deog Hwan (2019). Gut microbiome modulation based on probiotic application for anti-obesity: A review on efficacy and validation. *Microorganisms.7*(10), 456.

Barbieri, Federica, Montanari, Chiara, Gardini, Fausto, & Tabanelli, Giulia. (2019). Biogenic amine production by lactic acid bacteria: A Review. *Foods, 8*(1), 17.

Baxter, Nielson T, Schmidt, Alexander W, Venkataraman, Arvind, Kim, Kwi S, Waldron, Clive, & Schmidt, Thomas M (2019). Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. *MBio*. *10*(1).

Bellali, Sara, Lagier, Jean-Christophe, Raoult, Didier, Khalil, Bou, & Yaacoub, Jacques (2019). Among live and dead bacteria, the optimization of sample collection and processing remains essential in recovering gut microbiota components. *Frontiers in microbiology*.*10*, 1606.

Benkerroum, Noreddine. (2016). Biogenic amines in dairy products: origin, incidence, and control means. *Comprehensive Reviews in Food Science and Food Safety, 15*(4), 801-826.

Bertelli, Claire, Laird, Matthew R, Williams, Kelly P, Group, Simon Fraser University Research Computing, Lau, Britney Y, Hoad, Gemma, Winsor, Geoffrey L, & Brinkman, Fiona SL (2017). IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic acids research. 45*(W1), W30-W35.

Beyerlein, Andreas, Kusian, Dennis, Ziegler, Anette-Gabriele, Schaffrath-Rosario, Angelika, & von Kries, Rüdiger. (2014). Classification tree analyses reveal limited potential for early targeted prevention against childhood overweight. *Obesity, 22*(2), 512-517.

Bhattarai, Rewati Raman, Das, Suman Kumar Lal (2013). Scientific study on indigenous technology of Dahi making of eastern Nepal. *Journal of Food Processing, & Technology*. *4*(8).

Bhattarai, Rewati Raman, Gautam, Nawaraj, Nawaz, Malik Adil, & Das, Suman Kumar Lal. (2016). Isolation and identification of dominant lactic acid bacteria from dahi: an indigenous dairy product of Nepal Himalayas. *The Journal of Microbiology, Biotechnology and Food Sciences, 5*(4), 358.

Bhola, Jinal, Bhadekar, Rama (2019). *Invitro* synergistic activity of lactic acid bacteria against multi-drug resistant *staphylococci. BMC complementary, & medicine, alternative.19*(1), 1-8.

Bintsis, T (2018). Lactic acid bacteria: their applications in foods. *Bacteriol Mycol*. *6*(2), 89-94.

Bintsis, Thomas. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS Microbiology, 4*(4), 665.

Blaut, Michael. (2015). Gut microbiota and energy balance: role in obesity. *Proceedings of the Nutrition Society, 74*(3), 227-234.

Blaya, J, Barzideh, Z, & LaPointe, G. (2018). Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment. *Journal of dairy science, 101*(4), 3611-3629.

Bliss, Edward S, & Whiteside, Eliza (2018). The gut-brain axis, the human gut microbiota and their integration in the development of obesity. *Frontiers in physiology*. *9*, 900.

Blüher, Matthias (2019). Obesity: global epidemiology and pathogenesis. *Nature Reviews Endocrinology.15*(5), 288-298.

Bohn, Torsten, Carriere, Frèderic, Day, L, Deglaire, Amélie, Egger, Lotti, Freitas, Daniela, Golding, Matt, Le Feunteun, Steven, Macierzanka, Adam, Ménard, Olivia (2018). Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static in vitro digestion models?*, Critical reviews in food science, & nutrition*. *58*(13), 2239-2261.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics, 30*(15), 2114-2120. doi:10.1093/bioinformatics/btu170

Booth, Frank W, Roberts, Christian K, Thyfault, John P, Ruegsegger, Gregory N, & Toedebusch, Ryan G (2017). Role of inactivity in chronic diseases: evolutionary insight and pathophysiological mechanisms. *Physiological reviews 97*(4), 1351-1402.

Borriello, SP, Hammes, WP, Holzapfel, W, Marteau, P, Schrezenmeir, J, Vaara, M, & Valtonen, Ville (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. *Clinical infectious diseases 36*(6), 775-780.

Bouchard, Claude, Tremblay, Angelo, Després, Jean-Pierre, Nadeau, André, Lupien, Paul J, Thériault, Germain, Dussault, Jean, Moorjani, Sital, Pinault, Sylvie, & Fournier, Guy. (1990). The response to long-term overfeeding in identical twins. *New England Journal of Medicine, 322*(21), 1477-1482.

Boutagy, Nabil E, McMillan, Ryan P, Frisard, Madlyn I, & Hulver, Matthew W. (2016). Metabolic endotoxemia with obesity: Is it real and is it relevant? *Biochimie, 124*, 11-20.

Bover-Cid, Sara, & Holzapfel, Wilhelm Heinrich (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *International journal of food microbiology*. *53*(1), 33-41.

Bozoudi, Despina, Kotzamanidis, Charalambos, Hatzikamari, Magdalini, Tzanetakis, Nikolaos, Menexes, George, & Litopoulou-Tzanetaki, Evanthia. (2015). A comparison for acid production, proteolysis, autolysis and inhibitory properties of lactic acid bacteria from fresh and mature Feta PDO Greek cheese, made at three different mountainous areas. *International journal of food microbiology, 200*, 87-96.

Bray, GA, Kim, KK, Wilding, JPH, & Federation, World Obesity. (2017a). Obesity: a chronic relapsing progressive disease process. A position statement of the World Obesity Federation. *Obesity reviews, 18*(7), 715-723.

Bray, GA, Kim, KK, Wilding, JPH, & Reviews, World Obesity Federation (2017b). Obesity: a chronic relapsing progressive disease process. A position statement of the World Obesity Federation. *Obesity*. *18*(7), 715-723.

Brodkorb, André, Egger, Lotti, Alminger, Marie, Alvito, Paula, Assunção, Ricardo, Ballance, Simon, Bohn, Torsten, Bourlieu-Lacanal, Claire, Boutrou, Rachel, & Carrière, Frédéric (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature protocols 14*(4), 991-1014.

Burke, Niamh, Zacharski, Krzysztof A, Southern, Mark, Hogan, Paul, Ryan, Michael P, & Adley, Catherine C. (2018). The Dairy Industry: Process, Monitoring, Standards, and Quality. In *Descriptive Food Science*: IntechOpen.

Buron-Moles, Gemma, Chailyan, Anna, Dolejs, Igor, Forster, Jochen, Mikš, Marta Hanna. (2019). Uncovering carbohydrate metabolism through a genotype-phenotype association study of 56 lactic acid bacteria genomes. *Applied microbiology, & biotechnology 103*(7), 3135-3152.

Butland, Bryony, Jebb, Susan, Kopelman, Peter, McPherson, Klim, Thomas, Sandy, Mardell, Jane, & Parry, Vivienne. (2007). Foresight. Tackling obesities: future choices. Project report. *Foresight. Tackling obesities: future choices. Project report.*

Callaway, Leonie K, McIntyre, H David, Barrett, Helen L, Foxcroft, Katie, Tremellen, Anne, Lingwood, Barbara E, Tobin, Jacinta M, Wilkinson, Shelley, Kothari, Alka, & Morrison, Mark (2019). Probiotics for the prevention of gestational diabetes mellitus in overweight and obese women: findings from the SPRING double-blind randomized controlled trial. *Diabetes Care. 42*(3), 364-371.

Campana, Raffaella, van Hemert, Saskia, & Baffone, Wally (2017). Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut pathogens. 9*(1), 1-12.

Cani, Patrice D (2012). Crosstalk between the gut microbiota and the endocannabinoid system: impact on the gut barrier function and the adipose tissue. *Clinical Microbiology, & Infection 18*, 50-53.

Cani, Patrice D, Bibiloni, Rodrigo, Knauf, Claude, Waget, Aurélie, Neyrinck, Audrey M, Delzenne, Nathalie M, & Burcelin, Rémy. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. *Diabetes, 57*(6), 1470-1481.

Cani, Patrice D, & Delzenne, Nathalie M. (2011). The gut microbiome as therapeutic target. *Pharmacology & therapeutics, 130*(2), 202-212.

Cani, Patrice D, Possemiers, Sam, Van de Wiele, Tom, Guiot, Yves, Everard, Amandine, Rottier, Olivier, Geurts, Lucie, Naslain, Damien, Neyrinck, Audrey, & Lambert, Didier M (2009). Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut*. *58*(8), 1091-1103.

Carabotti, Marilia, Scirocco, Annunziata, Maselli, Maria Antonietta, & Severi, Carola (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of gastroenterology: quarterly publication of the Hellenic Society of Gastroenterology*. *28*(2), 203.

Carden, Trevor J, & Carr, Timothy P. (2013). Food availability of glucose and fat, but not fructose, increased in the US between 1970 and 2009: analysis of the USDA food availability data system. *Nutrition journal, 12*(1), 130.

Chambers, Edward S, Preston, Tom, Frost, Gary, & Morrison, Douglas J (2018). Role of gut microbiota-generated short-chain fatty acids in metabolic and cardiovascular health. *Current nutrition reports. 7*(4), 198-206.

Chen, Taohong, Wang, Leli, Li, Qinxin, Long, Yingjie, Lin, Yuming, Yin, Jie, Zeng, Yan, Huang, Le, Yao, Tingyu, & Abbasi, Muhammad Nazeer (2020). Functional probiotics of lactic acid bacteria from Hu sheep milk. *BMC microbiology. 20*(1), 1-12.

Cheng, Ching-Feng, Ku, Hui-Chen, & Lin, Heng (2018). PGC-1α as a pivotal factor in lipid and metabolic regulation. *International journal of molecular sciences. 19*(11), 3447.

Chiang, John YL, Pathak, Preeti, Liu, Hailiang, Donepudi, Ajay, Ferrell, Jessica, & Boehme, Shannon (2017). Intestinal farnesoid X receptor and Takeda G protein couple receptor 5 signaling in metabolic regulation. *Digestive Diseases. 35*(3), 241-245.

Choi, Ah-Rang, Patra, Jayanta Kumar, Kim, Wang June, & Kang, Seok-Seong (2018). Antagonistic activities and probiotic potential of lactic acid bacteria derived from a plant-based fermented food. *Frontiers in microbiology. 9*, 1963.

Choi, Jongwoo, Sabikhi, Latha, Hassan, Ashraf, & Anand, Sanjeev. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12.

Chun-Sai-Er Wang, Wen-Bin, Li, Hong-Ying Wang, Yi-Ming Ma, Xin-Hua Zhao, & Hong Yang, Jia-Ming Qian (2018). VSL# 3 can prevent ulcerative colitis-associated carcinogenesis in mice. *World journal of gastroenterology. 24*(37), 4254.

Cieplak, Tomasz, Wiese, Maria, Nielsen, Sebastian, Van de Wiele, Tom, van den Berg, Frans, & Nielsen, Dennis Sandris (2018). The smallest intestine (TSI)—a low volume *in vitro* model of the small intestine with increased throughput. *FEMS microbiology letters*. *365*(21), fny231.

Cissé, Hama, Kagambèga, Boureima, Sawadogo, Adama, Tankoano, Abel, Sangaré, Gaston, Traoré, Yves, Ouoba, Ivette Irène Labia, & Savadogo, Aly (2019). Molecular characterization of Bacillus, lactic acid bacteria and yeast as potential probiotic isolated from fermented food. *Scientific African. 6*, e00175.

Clemente-Postigo, Mercedes, Oliva-Olivera, Wilfredo, Coin-Aragüez, Leticia, Ramos-Molina, Bruno, Giraldez-Perez, Rosa María, Lhamyani, Said, Alcaide-Torres, Juan, Perez-Martinez, Pablo, El Bekay, Rajaa, Cardona, Fernando (2019). Metabolic endotoxemia

promotes adipose dysfunction and inflammation in human obesity. *American Journal of Physiology-Endocrinology, & Metabolism*. *316*(2), E319-E332.

Cohen, Deborah A. (2008). Neurophysiological pathways to obesity: below awareness and beyond individual control. *Diabetes, 57*(7), 1768-1773.

Collaboration, NCD Risk Factor. (2016). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19· 2 million participants. *The Lancet, 387*(10026), 1377-1396.

Collaborators, GBD Obesity. (2017). Health effects of overweight and obesity in 195 countries over 25 years. *New England Journal of Medicine, 377*(1), 13-27.

Collins, Fergus WJ, O'Connor, Paula M, O'Sullivan, Orla, Gómez-Sala, Beatriz, Rea, Mary C, Hill, Colin, & Ross, R Paul (2017). Bacteriocin Gene-Trait matching across the complete Lactobacillus Pan-genome. *Scientific reports. 7*(1), 1-14.

Cook, Michael T, Tzortzis, George, Charalampopoulos, Dimitris, & Khutoryanskiy, Vitaliy V (2012). Microencapsulation of probiotics for gastrointestinal delivery. *Journal of controlled release*. *162*(1), 56-67.

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2007). Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. *Microbiology, 153*(1), 291-299.

Crovesy, Louise, Masterson, Daniele, & Rosado, Eliane Lopes (2020). Profile of the gut microbiota of adults with obesity: a systematic review. *European journal of clinical nutrition*. *74*(9), 1251-1262.

D O'Brien, Phillipe, Hinder, Lucy M, Callaghan, Brian C, & Feldman, Eva L (2017). Neurological consequences of obesity. *The Lancet Neurology. 16*(6), 465-477.

da Silva, Tatiane Ferreira, Casarotti, Sabrina Neves, de Oliveira, Gislane Lelis Vilela, Penna, Ana Lúcia Barretto (2021). The impact of probiotics, prebiotics, and synbiotics on the biochemical, clinical, and immunological markers, as well as on the gut microbiota of obese hosts. *Critical reviews in food science, & nutrition. 61*(2), 337-355.

Daeschel, Mark A, & Fleming, Henry P. (1984). Selection of lactic acid bacteria for use in vegetable fermentations. *Food microbiology, 1*(4), 303-313.

Dalgaard, Kevin, Landgraf, Kathrin, Heyne, Steffen, Lempradl, Adelheid, Longinotto, John, Gossens, Klaus, Ruf, Marius, Orthofer, Michael, Strogantsev, Ruslan, & Selvaraj, Madhan (2016). Trim28 haploinsufficiency triggers bi-stable epigenetic obesity. *Cell. 164*(3), 353-364.

Dar, Lior, Tiosano, Shmuel, Watad, Abdulla, Bragazzi, Nicola Luigi, Zisman, Devi, Comaneshter, Doron, Cohen, Arnon, & Amital, Howard. (2018). Are obesity and rheumatoid arthritis interrelated? *International journal of clinical practice, 72*(1), e13045.

Daroonpunt, Rungsima, Tanaka, Naoto, Uchino, MASATAKA, & Tanasupawat, SOMBOON. (2018). Characterization and screening of lipolytic bacteria from Thai fermented fish. *Sains Malaysiana, 47*(1), 91-97.

Davidson, Barrie E, Kordias, Nancy, Dobos, Marian, & Hillier, Alan J (1996). Genomic organization of lactic acid bacteria. *Antonie Van Leeuwenhoek. 70*(2), 161-183.

de Almeida Júnior, Washington Luiz Gonçalves, da Silva Ferrari, Íris, de Souza, Jane Viana, da Silva, Carla Daiane Andrade, da Costa, Mateus Matiuzzi, & Dias, Francesca Silva (2015). Characterization and evaluation of lactic acid bacteria isolated from goat milk. *Food Control*. *53*, 96-103.

De Lorenzo, Antonino, Costacurta, Micaela, Merra, Giuseppe, Gualtieri, Paola, Cioccoloni, Giorgia, Marchetti, Massimiliano, Varvaras, Dimitrios, Docimo, Raffaella, & Di Renzo, Laura (2017). Can psychobiotics intake modulate psychological profile and body composition of women affected by normal weight obese syndrome and obesity? A double blind randomized clinical trial. *Journal of translational medicine. 15*(1), 1-12.

de Melo Pereira, Gilberto Vinícius, de Oliveira Coelho, Bruna, Júnior, Antonio Irineudo Magalhães, Thomaz-Soccol, Vanete, & Soccol, Carlos Ricardo (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnology advances. 36*(8), 2060-2076.

DeGruttola, Arianna K, Low, Daren, Mizoguchi, Atsushi, & Mizoguchi, Emiko (2016). Current understanding of dysbiosis in disease in human and animal models. *Inflammatory bowel diseases*. *22*(5), 1137-1150.

Delgado, Amélia Martins, Parisi, Salvatore, & Almeida, Maria Daniel Vaz. (2017). Milk and dairy products. In *Chemistry of the Mediterranean Diet* (pp. 139-176): Springer.

Den Besten, Gijs, van Eunen, Karen, Groen, Albert K, Venema, Koen, Reijngoud, Dirk-Jan, & Bakker, Barbara M (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research. 54*(9), 2325-2340.

Díaz-Ruiz, Gloria, Guyot, JeanPierre, Ruiz-Teran, Francisco, Morlon-Guyot, Juliette, & Wacher, Carmen. (2003). Microbial and physiological characterization of weakly amylolytic but fast-growing lactic acid bacteria: a functional role in supporting microbial diversity in pozol, a Mexican fermented maize beverage. *Appl. Environ. Microbiol., 69*(8), 4367-4374.

Dinçer, Emine, & Kıvanç, Merih. (2018). Lipolytic Activity of Lactic Acid Bacteria Isolated from Turkish Pastırma. *Anadolu Üniversitesi Bilim ve Teknoloji Dergisi-C Yaşam Bilimleri Ve Biyoteknoloji, 7*(1), 12-19.

Dlamini, Ziyanda C, Langa, Rashwahla LS, Aiyegoro, Olayinka A, Okoh, Anthony I (2019). Safety evaluation and colonisation abilities of four lactic acid bacteria as future probiotics. *Probiotics, & proteins, antimicrobial. 11*(2), 397-402.

Do, Moon Ho, Lee, Eunjung, Oh, Mi-Jin, Kim, Yoonsook, & Park, Ho-Young (2018). High-glucose or-fructose diet cause changes of the gut microbiota and metabolic disorders in mice without body weight change. *Nutrients. 10*(6), 761.

Dora, Pereira, & Glenn, Gibson. (2002). Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. *Appl. Environ. Microbiol., 68*(9), 4689-4693.

Doron, Shira, & Snydman, David R (2015). Risk and safety of probiotics. *Clinical Infectious Diseases*. *60*(suppl_2), S129-S134.

Duan, Yehui, Zeng, Liming, Zheng, Changbing, Song, Bo, Li, Fengna, Kong, Xiangfeng, & Xu, Kang. (2018). Inflammatory links between high fat diets and diseases. *Frontiers in Immunology, 9*.

Dubé, Philip E, Brubaker, Patricia L (2007). Frontiers in glucagon-like peptide-2: multiple actions, multiple mediators. *American Journal of Physiology-Endocrinology, & Metabolism. 293*(2), E460-E465.

Dumitru, Calin D, Ceci, Jeffrey D, Tsatsanis, Christos, Kontoyiannis, Dimitris, Stamatakis, Konstantinos, Lin, Jun-Hsiang, Patriotis, Christos, Jenkins, Nancy A, Copeland, Neal G, & Kollias, George. (2000). TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell, 103*(7), 1071-1083.

Durazo-Arvizu, Ramón A, Luke, Amy, Cooper, Richard S, Cao, Guichan, Dugas, Lara, Adeyemo, Adebowale, Boyne, Michael, & Forrester, Terrence. (2008). Rapid increases in obesity in Jamaica, compared to Nigeria and the United States. *BMC public health, 8*(1), 133.

Duysburgh, Cindy, Van den Abbeele, Pieter, & Marzorati, Massimo (2018). SHIME®: an advanced in *vitro* technology platform for studying the mode-of-action of probiotics in the gastrointestinal tract. *International Journal on Nutraceuticals, Functional Foods and Novel Foods.*

Egger, Lotti, Ménard, Olivia, Baumann, Christian, Duerr, Desirée, Schlegel, Patrick, Stoll, Peter, Vergères, Guy, Dupont, Didier, & Portmann, Reto (2019). Digestion of milk proteins: Comparing static and dynamic in vitro digestion systems with *in vivo* data. *Food Research International*. *118*, 32-39.

Eknoyan, Garabed (2006). A history of obesity, or how what was good became ugly and then bad. *Advances in chronic kidney disease*.*13*(4), 421-427.

Eloe-Fadrosh, Emiley A, Brady, Arthur, Crabtree, Jonathan, Drabek, Elliott F, Ma, Bing, Mahurkar, Anup, Ravel, Jacques, Haverkamp, Miriam, Fiorino, Anne-Maria, & Botelho, Christine (2015). Functional dynamics of the gut microbiome in elderly people during probiotic consumption. *MBio. 6*(2), e00231-00215.

Eshaque, Tamannyat Binte. (2018). *Development of Lactic Acid bacteria starter culture for probiotic yogurt* (Doctoral dissertation).

Faccia, Michele, D'Alessandro, Angela Gabriella, Summer, Andrea, & Hailu, Yonas %J Animals. (2020). Milk products from minor dairy species: A review. *10*(8), 1260.

Feng, Xiao-wei, Ding, Wen-ping, Xiong, Ling-yun, Guo, Liang, Sun, Jia-ming, & Xiao, Peng. (2018). Recent Advancements in Intestinal Microbiota Analyses: A Review for Non-Microbiologists. *Current medical science, 38*(6), 949-961.

Fernandes, JJDR, Su, W, Rahat-Rozenbloom, S, Wolever, TMS, Comelli, EM (2014). Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutrition, & diabetes. 4*(6), e121-e121.

Field, Des, Ross, R Paul, & Hill, Colin (2018). Developing bacteriocins of lactic acid bacteria into next generation biopreservatives. *Current Opinion in Food Science*. *20*, 1-6.

Flint, Stuart W. (2019). The complexity of obesity. *The Lancet Diabetes & Endocrinology*.

Foong, FCF, Cheng, KJ, Abdullah, N, Ho, YW, Jalaludin, S, Sugiura, M, Nagamine, T, Arakaki, C, & Kudo, H. (1997). Selective isolation and characterization of cellulolytic bacteria by cellulose enrichment method from the rumen of ruminants. *JIRCAS Journal for Scientific Papers (Japan)*.

Formisano, M, Coppola, S, Percuoco, G, Percuoco, S, & Zoina, A. (1974). Lipolytic activity of Streptococcus thermophilus and of Lactobacillus bulgaricus. *Nuovi annali d'igiene e microbiologia, 25*(3), 223.

Fox, Ashley, Feng, Wenhui, & Asal, Victor. (2019). What is driving global obesity trends? Globalization or "modernization"? *Globalization and health, 15*(1), 32.

Galperin, M. Y., Makarova, K. S., Wolf, Y. I., & Koonin, E. V. (2015). Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res, 43* (Database issue), D261-269. doi:10.1093/nar/gku1223

García-Burgos, María, Moreno-Fernández, Jorge, Alférez, María JM, Díaz-Castro, Javier, & López-Aliaga, Inmaculada (2020). New perspectives in fermented dairy products and their health relevance. *Journal of Functional Foods*. *72*, 104059.

Garg, Sarita, Zheng, Junying, Wang, Junru, Authier, Simon, Pouliot, Mylene, & Hauer-Jensen, Martin. (2016). Segmental differences in radiation-induced alterations of tight junction-related proteins in non-human primate jejunum, ileum and colon. *Radiation research 185*(1), 50-59.

Georgieva, Ralitsa, Yocheva, Lyubomira, Tserovska, Lilia, Zhelezova, Galina, Stefanova, Nina, Atanasova, Akseniya, Danguleva, Antonia, Ivanova, Gergana, Karapetkov, Nikolay, Rumyan, Nevenka (2015). Antimicrobial activity and antibiotic susceptibility of Lactobacillus and Bifidobacterium spp. intended for use as starter and probiotic cultures. *Biotechnology, & Equipment, Biotechnological. 29*(1), 84-91.

Geserick, Mandy, Vogel, Mandy, Gausche, Ruth, Lipek, Tobias, Spielau, Ulrike, Keller, Eberhard, Pfäffle, Roland, Kiess, Wieland, & Körner, Antje. (2018). Acceleration of BMI in early childhood and risk of sustained obesity. *New England Journal of Medicine, 379*(14), 1303-1312.

Gesta, Stephane, Blüher, Matthias, Yamamoto, Yuji, Norris, Andrew W, Berndt, Janin, Kralisch, Susan, Boucher, Jeremie, Lewis, Choy, & Kahn, C Ronald (2006). Evidence for a role of

developmental genes in the origin of obesity and body fat distribution. *Proceedings of the National Academy of Sciences. 103*(17), 6676-6681.

Ghosh, J, & Rajorhia, GS (1990). Selection of starter culture for production of indigenous fermented milk product (Misti dahi). *Le Lait. 70*(2), 147-154.

Ghosh, Tamoghna, Beniwal, Arun, Semwal, Anupama, & Navani, Naveen Kumar (2019). Mechanistic insights into probiotic properties of lactic acid bacteria associated with ethnic fermented dairy products. *Frontiers in microbiology. 10*, 502.

Gilbert-Diamond, Diane, Emond, Jennifer A, Lansigan, Reina K, Rapuano, Kristina M, Kelley, William M, Heatherton, Todd F, & Sargent, James D. (2017). Television food advertisement exposure and FTO rs9939609 genotype in relation to excess consumption in children. *International journal of obesity, 41*(1), 23.

Giovanna, Muscogiuri, Cantone, Elena, Sara, Cassarano, Dario, Tuccinardi, Luigi, Barrea, Savastano, Silvia, & Annamaria, Colao (2019). Gut microbiota: a new path to treat obesity. *International Journal of Obesity Supplements. 9*(1), 10-19.

Giraud, Eric, Champailler, Alain, & Raimbault, Maurice. (1994). Degradation of raw starch by a wild amylolytic strain of Lactobacillus plantarum. *Appl. Environ. Microbiol., 60*(12), 4319-4323.

González, Sacristán, Noelia, Arenas, Ricardo, Fresno, José M, & Tornadijo, M Eugenia. (2010). Enzymatic activity of lactic acid bacteria (with antimicrobial properties) isolated from a traditional Spanish cheese. *Food microbiology, 27*(5), 592-597.

Grissa, Ibtissem, Vergnaud, Gilles, & Pourcel, Christine (2007a). The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC bioinformatics. 8*(1), 1-10.

Grissa, Ibtissem, Vergnaud, Gilles, & Pourcel, Christine (2007b). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic acids research. 35*(suppl_2), W52-W57.

Grissa, Ibtissem, Vergnaud, Gilles, & Pourcel, Christine (2008). CRISPRcompar: a website to compare clustered regularly interspaced short palindromic repeats. *Nucleic acids research. 36*(suppl_2), W145-W148.

Grover, Sunita, Rashmi, Hogarehalli Mallapa, Srivastava, Anil Kumar, & Batish, Virender Kumar. (2012). Probiotics for human health–new innovations and emerging trends. In: Springer.

Guevara-Cruz, Martha, Flores-López, Adriana G, Aguilar-López, Miriam, Sánchez-Tapia, Mónica, Medina-Vera, Isabel, Díaz, Daniel, Tovar, Armando R, & Torres, Nimbe (2019). Improvement of lipoprotein profile and metabolic endotoxemia by a lifestyle intervention that modifies the gut microbiota in subjects with metabolic syndrome. *Journal of the American Heart Association. 8*(17), e012401.

Guo, Xiao-Hua, Kim, Jong-Man, Nam, Hyang-Mi, Park, Shin-Young, & Kim, Jae-Myung. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. *Anaerobe, 16*(4), 321-326.

Gupta, Radhey S (2000). The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiology Reviews*. *24*(4), 367-402.

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics, 29*(8), 1072-1075. doi:10.1093/bioinformatics/btt086

Haghshenas, Babak, Nami, Yousef, Almasi, Ali, Abdullah, Norhafizah, Radiah, Dayang, Rosli, Rozita, Barzegari, Abolfazl, & Khosroushahi, Ahmad Yari. (2017). Isolation and characterization of probiotics from dairies. *Iranian journal of microbiology, 9*(4), 234.

Hales, Craig M, Carroll, Margaret D, Fryar, Cheryl D, & Ogden, Cynthia L. (2020). Prevalence of obesity and severe obesity among adults: United States, 2017–2018.

Hall, Kevin D, Guo, Juen, Dore, Michael, & Chow, Carson C. (2009). The progressive increase of food waste in America and its environmental impact. *PloS one, 4*(11), e7940.

Hammes, Walter P, & Vogel, Rudi F. (1995). The genus lactobacillus. In *The genera of lactic acid bacteria* (pp. 19-54): Springer.

Han, Shengyi, Lu, Yanmeng, Xie, Jiaojiao, Fei, Yiqiu, Zheng, Guiwen, Wang, Ziyuan, Liu, Jie, Lv, Longxian, Ling, Zongxin, Berglund, Björn (2021). Probiotic gastrointestinal transit and colonization after oral administration: A long journey. *Frontiers in Cellular, & Microbiology, Infection. 11*, 102.

Hanchi, Hasna, Mottawea, Walid, Sebei, Khaled, & Hammami, Riadh. (2018a). The genus Enterococcus: Between probiotic potential and safety concerns—An update. *Frontiers in Microbiology, 9*.

Hanchi, Hasna, Mottawea, Walid, Sebei, Khaled, & Hammami, Riadh (2018b). The genus *Enterococcus*: between probiotic potential and safety concerns—an update. *Frontiers in microbiology. 9*, 1791.

Hankin, Lester, & Anagnostakis, Sandra L. (1977). Solid media containing carboxymethylcellulose to detect Cx cellulase activity of micro-organisms. *Microbiology, 98*(1), 109-115.

Hanzelmann, Dennis, Joo, Hwang-Soo, Franz-Wachtel, Mirita, Hertlein, Tobias, Stevanovic, Stefan, Macek, Boris, Wolz, Christiane, Götz, Friedrich, Otto, Michael, & Kretschmer, Dorothee (2016). Toll-like receptor 2 activation depends on lipopeptide shedding by bacterial surfactants. *Nature communications. 7*(1), 1-11.

Hardin, Jessica, McLennan, Amy K, & Brewis, Alexandra. (2018). Body size, body norms and some unintended consequences of obesity intervention in the Pacific islands. *Annals of human biology, 45*(3), 285-294.

Harris, Randall E. (2019). *Epidemiology of chronic disease: global perspectives*: Jones & Bartlett Learning.

Harun-ur-Rashid, Md, Togo, Kaname, Ueda, Minoru, & Miyamoto, Taku. (2007a). Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk Dahi in Bangladesh. *World Journal of Microbiology and Biotechnology, 23*(1), 125-133.

Harun-ur-Rashid, Md, Togo, Kaname, Ueda, Minoru, & Miyamoto, Taku (2007b). Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk "Dahi" in Bangladesh. *Pakistan Journal of Nutrition. 6*, 647-652.

Harun-ur-Rashid, Md, Togo, Kaname, Ueda, Minoru, Miyamoto, Taku (2007c). Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk Dahi in Bangladesh. *World Journal of Microbiology, & Biotechnology. 23*(1), 125-133.

Harzallah, Daoud, & Belhadj, Hani (2013). Lactic acid bacteria as probiotics: characteristics, selection criteria and role in immunomodulation of human GI muccosal barrier. *Kongo M.* 197-216.

Hasan, Nihal, & Yang, Hongyi (2019). Factors affecting the composition of the gut microbiota, and its modulation. *PeerJ. 7*, e7502.

Hassanzadazar, Hassan, Ehsani, Ali, Mardani, Karim, & Hesari, Javad. (2012). *Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese.* Paper presented at the Veterinary Research Forum.

Hawkes, Corina, Jewell, J, & Allen, K (2013). A food policy package for healthy diets and the prevention of obesity and diet-related non-communicable diseases: the NOURISHING framework. *Obesity reviews. 14*, 159-168.

Hayes, Christina L, Dong, Jasmine, Galipeau, Heather J, Jury, Jennifer, McCarville, Justin, Huang, Xianxi, Wang, Xuan-Yu, Naidoo, Avee, Anbazhagan, Arivarasu N, & Libertucci, Josie (2018). Commensal microbiota induces colonic barrier structure and functions that contribute to homeostasis. *Scientific reports. 8*(1), 1-14.

Heidlauf, Thomas, & Röhrle, Oliver (2014). A multiscale chemo-electro-mechanical skeletal muscle model to analyze muscle contraction and force generation for different muscle fiber arrangements. *Frontiers in physiology. 5*, 498.

Heiker, John T, Kunath, Anne, Kosacka, Joanna, Flehmig, Gesine, Knigge, Anja, Kern, Matthias, Stumvoll, Michael, Kovacs, Peter, Blüher, Matthias, & Klöting, Nora (2014). Identification of genetic loci associated with different responses to high-fat diet-induced obesity in C57BL/6N and C57BL/6J substrains. *Physiological genomics. 46*(11), 377-384.

Hernandez-Sanabria, Emma, Heiremans, Evelien, Arroyo, Marta Calatayud, Props, Ruben, Leclercq, Laurent, Snoeys, Jan, Van de Wiele, Tom (2020). Short-term supplementation of celecoxib-shifted butyrate production on a simulated model of the gut microbial ecosystem and ameliorated in vitro inflammation. *NPJ biofilms, & microbiomes. 6*(1), 1-12.

Hersoug, Lars-Georg, Møller, Peter, & Loft, Steffen (2018). Role of microbiota-derived lipopolysaccharide in adipose tissue inflammation, adipocyte size and pyroptosis during obesity. *Nutrition research reviews. 31*(2), 153-163.

Hess, Martin E, Hess, Simon, Meyer, Kate D, Verhagen, Linda AW, Koch, Linda, Brönneke, Hella S, Dietrich, Marcelo O, Jordan, Sabine D, Saletore, Yogesh, & Elemento, Olivier. (2013). The fat mass and obesity associated gene (Fto) regulates activity of the dopaminergic midbrain circuitry. *Nature neuroscience, 16*(8), 1042.

Ho, Jason Chen Kok, & Yin Sze, Lim. (2018). Isolation, identification and characterization of enzyme-producing lactic acid bacteria from traditional fermented foods. *Bioscience Horizons: The International Journal of Student Research, 11*.

Holst, Jens Juul (2007). The physiology of glucagon-like peptide 1. *Physiological reviews. 87*(4), 1409-1439.

Hong, Young-Rock, Pavela, Gregory, Lee, Alexandra M, Williamson, Victoria G, & Cardel, Michelle I. (2019). Satisfaction with Health Care Among Individuals with Overweight and Obesity: A Nationally Representative Cross-sectional Study. *Journal of general internal medicine*, 1-3.

Hoshi, Namiko, Inoue, Jun, Sasaki, Daisuke, Sasaki, Kengo (2021). The Kobe University Human Intestinal Microbiota Model for gut intervention studies. *Applied Microbiology, & Biotechnology*. 1-8.

Hou, Qiangchuan, Zhao, Feiyan, Liu, Wenjun, Lv, Ruirui, Khine, Wei Wei Thwe, Han, Jia, Sun, Zhihong, Lee, Yuan-Kun, & Zhang, Heping (2020). Probiotic-directed modulation of gut microbiota is basal microbiome dependent. *Gut microbes*. *12*(1), 1736974.

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., Rattei, T., Mende, D. R., Sunagawa, S., Kuhn, M., Jensen, L. J., von Mering, C., & Bork, P. (2016). eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res, 44*(D1), D286-293. doi:10.1093/nar/gkv1248

Hui, Yan, Tamez-Hidalgo, Paulina, Cieplak, Tomasz, Satessa, Gizaw Dabessa, Kot, Witold, Kjærulff, Søren, Nielsen, Mette Olaf, Nielsen, Dennis Sandris, Krych, Lukasz (2021). Supplementation of a lacto-fermented rapeseed-seaweed blend promotes gut microbial-and gut immune-modulation in weaner piglets. *Journal of Animal Science, & Biotechnology*. *12*(1), 1-14.

Hummel, Anja S, Hertel, Christian, Holzapfel, Wilhelm H, & Franz, Charles MAP. (2007). Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Applied & Environmental Microbiology. 73*(3), 730-739.

Hungerford, James M. (2010). Scombroid poisoning: a review. *Toxicon, 56*(2), 231-243.

Igam, (2019). Gastrointestinal tract 4: anatomy and role of the jejunum and ileum. Y *Nurs Times. 115*(9), 43-46.

Imran, M, Desmasures, N, & Vernoux, J-P. (2010). From undefined red smear cheese consortia to minimal model communities both exhibiting similar anti-listerial activity on a cheese-like matrix. *Food microbiology, 27*(8), 1095-1103.

Imran, Muhammad, Bré, Jean-Michel, Guéguen, Marielle, Vernoux, Jean-Paul, & Desmasures, Nathalie. (2013). Reduced growth of Listeria monocytogenes in two model cheese microcosms is not associated with individual microbial strains. *Food microbiology, 33*(1), 30-39.

Jacobsen, Natashia Mai Yde, Nedergaard, Hanne Bjørn, Kock, Anette, Caglayan, Ibrahim, Laursen, Marie Munch, Lange, Eva-Marie, Marcial-Coba, Martín Sebastián, Bar-Shalom, Daniel, Nielsen, Dennis Sandris, & Müllertz, Anette (2021). Development of gastro-resistant coated probiotic granulates and evaluation of viability and release during simulated upper gastrointestinal transit. *LWT. 144*, 111174.

Jain, S, Yadav, H, Sinha, PR, Naito, Y, Marotta, F (2008). Dahi containing probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* has a protective effect against Salmonella enteritidis infection in mice. *International journal of immunopathology, & pharmacology. 21*(4), 1021-1029.

Jalili-Firoozinezhad, Sasan, Gazzaniga, Francesca S, Calamari, Elizabeth L, Camacho, Diogo M, Fadel, Cicely W, Bein, Amir, Swenor, Ben, Nestor, Bret, Cronce, Michael J, & Tovaglieri, Alessio (2019). A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. *Nature biomedical engineering. 3*(7), 520-531.

Jandhyala, Sai Manasa, Talukdar, Rupjyoti, Subramanyam, Chivkula, Vuyyuru, Harish, Sasikala, Mitnala, & Reddy, D Nageshwar (2015). Role of the normal gut microbiota. *World journal of gastroenterology: WJG. 21*(29), 8787.

Jang, Hyo-Min, Lee, Kyung-Eon, Lee, Hae-Ji, & Kim, Dong-Hyun (2018). Immobilization stress-induced Escherichia coli causes anxiety by inducing NF-κB activation through gut microbiota disturbance. *Scientific reports. 8*(1), 1-14.

Jatmiko, Yoga Dwi, Howarth, Gordon S, & Barton, Mary D. (2017). *Assessment of probiotic properties of lactic acid bacteria isolated from Indonesian naturally fermented milk.* Paper presented at the AIP Conference Proceedings.

Jehan, Shazia, Zizi, Ferdinand, Pandi-Perumal, Seithikurippu R, McFarlane, Samy I, Jean-Louis, Girardin, Myers, Alyson K (2020). Energy imbalance: obesity, associated comorbidities, prevention, management and public health implications. *Advances in obesity, weight management, & control. 10*(5), 146.

Jiao, Anran, Yu, Bing, He, Jun, Yu, Jie, Zheng, Ping, Luo, Yuheng, Luo, Junqiu, Mao, Xiangbing, Chen, Daiwen (2020). Short chain fatty acids could prevent fat deposition in pigs via regulating related hormones and genes. *Food, & Function. 11*(2), 1845-1855.

Jin, Young Hun, Lee, Jae Hoan, Park, Young Kyung, Lee, Jun-Hee, & Mah, Jae-Hyung. (2019). The occurrence of biogenic amines and determination of biogenic amine-producing lactic acid bacteria in Kkakdugi and Chonggak kimchi. *Foods, 8*(2), 73.

Joho, Shuji, Harada, Daisuke, Oda, Yoshitaka, Hirai, Tadakazu, Asanoi, Hidetsugu, & Inoue, Hiroshi (2018). Sympathoinhibitory effect is sustained even after removal of adaptive servo-ventilation in patients with heart failure. *Journal of the American College of Cardiology. 55*(10 supplement), a33. E320.

Joseph, Narcisse, Clayton, Jonathan B, Hoops, Susan L, Linhardt, Carter A, Mohd Hashim, Amalia, Mohd Yusof, Barakatun Nisak, Kumar, Suresh, & Amin Nordin, Syafinaz (2020). Alteration of the Gut Microbiome in Normal and Overweight School Children from Selangor with Lactobacillus Fermented Milk Administration. *Evolutionary Bioinformatics. 16*, 1176934320965943.

Kahan, Scott, & Manson, JoAnn E. (2019). Obesity Treatment, Beyond the Guidelines: Practical Suggestions for Clinical Practice. *Jama, 321*(14), 1349-1350.

Karatzi, Kalliopi, Moschonis, George, Choupi, Eurikleia, Manios, Yannis, Skenderi, Katerina P, Grammatikaki, Evangelia, Androutsos, Odysseas, Tanagra, Sofia, Koumpitski, Alexandra, & Siatitsa, Paraskevi-Eirini (2017). Late-night overeating is associated with smaller breakfast, breakfast skipping, and obesity in children: The Healthy Growth Study. *33*, *Nutrition.* 141-144.

Karlsson Videhult, Frida, Andersson, Yvonne, Öhlund, Inger, Stenlund, Hans, Hernell, Olle, West, Christina E (2015). Impact of probiotics during weaning on the metabolic and inflammatory profile: follow-up at school age. *International journal of food sciences, & nutrition. 66*(6), 686-691.

Kasubuchi, Mayu, Hasegawa, Sae, Hiramatsu, Takero, Ichimura, Atsuhiko, & Kimura, Ikuo (2015). Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients. 7*(4), 2839-2849.

Kathiriya, MR, Hati, S, Prajapati, JB, & Vekariya, YV. (2018). Assessment of in vitro probiotic potential of lactic acid bacteria. *Research & Reviews: Journal of Dairy Science and Technology, 5*(1), 17-30.

Katsowich, Naama, Elbaz, Netanel, Pal, Ritesh Ranjan, Mills, Erez, Kobi, Simi, Kahan, Tamar, & Rosenshine, Ilan (2017). Host cell attachment elicits posttranscriptional regulation in infecting enteropathogenic bacteria. *Science. 355*(6326), 735-739.

Katz, Marta, Medina, Roxana, Gonzalez, Silvia, & Oliver, Guillermo. (2002). Esterolytic and lipolytic activities of lactic acid bacteria isolated from ewe's milk and cheese. *Journal of food protection, 65*(12), 1997-2001.

Keisam, Santosh, Tuikhar, Ngangyola, Ahmed, Giasuddin, & Jeyaram, Kumaraswamy (2019). Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in the Northeast region of India. *International journal of food microbiology. 296*, 21-30.

Kell, Douglas B, & Pretorius, Etheresia (2015). On the translocation of bacteria and their lipopolysaccharides between blood and peripheral locations in chronic, inflammatory diseases: the central roles of LPS and LPS-induced cell death. *Integrative Biology. 7*(11), 1339-1377.

Khorshidian, Nasim, Yousefi, Mojtaba, Mortazavian, Amir M (2020). Fermented milk: The most popular probiotic food carrier. *Advances in Food, & Research, Nutrition*. *94*, 91-114.

Kim, Tae Jun, Makowski, Anna Christin, & von dem Knesebeck, Olaf. (2019). Obesity stigma in Germany and the United States–Results of population surveys. *PloS one, 14*(8).

Kim, Young-Hoon, Kim, Sae-Hun, Whang, Kwang-Youn, Kim, Young-Jun, Oh, Se-Jong (2008). Inhibition of Escherichia coli O157: H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. *Journal of microbiology, & biotechnology. 18*(7), 1278-1285.

Koh, Keumseok, Elder, Todd E, Grady, Sue C, Darden, Joe T, & Vojnovic, Igor. (2018). Explained and unexplained racial and regional inequality in obesity prevalence in the United States. *Ethnicity & health*, 1-14.

Kopelman, Peter G. (2000). Obesity as a medical problem. *nature, 404*(6778), 635.

Kothandaraman, S, Christy, R John, Varadarajan, A, & Gnanasekar, R (2019). Augmenting milk production in buffaloes by combating heat stress through strategic supplementation of drug based probiotics and immunomodulators. *The pharma innovation journal. 8*(1), 314-317.

Krasowska, Anna, & Sigler, Karel. (2014). How microorganisms use hydrophobicity and what does this mean for human needs? *Frontiers in cellular and infection microbiology, 4*, 112.

Krasowska, Anna, Sigler, Karel (2014). How microorganisms use hydrophobicity and what does this mean for human needs? *Frontiers in cellular, & microbiology, infection. 4*, 112.

Krishna Rao, Radha, Samak, Geetha (2013). Protection and restitution of gut barrier by probiotics: nutritional and clinical implications. *Current Nutrition, & Science, Food. 9*(2), 99-107.

Kriss, Michael, Hazleton, Keith Z, Nusbacher, Nichole M, Martin, Casey G, & Lozupone, Catherine A (2018). Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery. *Current opinion in microbiology. 44*, 34-40.

Krych, Łukasz, Castro-Mejía, Josué L, Forero-Junco, Laura M, Moesby, Daniel N, Mikkelsen, Morten B, Rasmussen, Morten A, Sykulski, Maciej, & Nielsen, Dennis S (2019). DNA enrichment and tagmentation method for species-level identification and strain-level differentiation using ON-rep-seq. *Communications biology. 2*(1), 1-10.

Kuo, Yung-Chih, & Yu, Hsin-Wei. (2011). Transport of saquinavir across human brain-microvascular endothelial cells by poly (lactide-co-glycolide) nanoparticles with surface poly-(γ-glutamic acid). *International journal of pharmaceutics, 416*(1), 365-375.

Kuźbicka, Karolina, & Rachoń, Dominik (2013). Bad eating habits as the main cause of obesity among children. *Pediatr Endocrinol Diabetes Metab. 19*(3), 106-110.

Kwoji, Iliya D, Aiyegoro, Olayinka A, Okpeku, Moses, & Adeleke, Matthew (2021). Multi-Strain Probiotics: Synergy among Isolates Enhances Biological Activities. *Biology. 10*(4), 322.

La Fata, Giorgio, Weber, Peter, Mohajeri, M Hasan (2018). Probiotics and the gut immune system: indirect regulation. *Probiotics, & proteins, antimicrobial. 10*(1), 11-21.

Ladero, Victor, Martín, María Cruz, Redruello, Begoña, Mayo, Baltasar, Flórez, Ana Belén, Fernández, María, & Alvarez, Miguel A. (2015). Genetic and functional analysis of biogenic amine production capacity among starter and non-starter lactic acid bacteria isolated from artisanal cheeses. *European Food Research and Technology, 241*(3), 377-383.

Lancaster, Graeme I, Langley, Katherine G, Berglund, Nils Anton, Kammoun, Helene L, Reibe, Saskia, Estevez, Emma, Weir, Jacquelyn, Mellett, Natalie A, Pernes, Gerard, & Conway, James RW (2018). Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipid-induced inflammation by reprogramming macrophage metabolism. *Cell metabolism. 27*(5), 1096-1110. e1095.

Landete, José Maria (2017). A review of food-grade vectors in lactic acid bacteria: from the laboratory to their application. *Critical reviews in biotechnology. 37*(3), 296-308.

Laslo, É, & György, É (2018). Evaluation of the microbiological quality of some dairy products. *Acta Universitatis Sapientiae, Alimentaria. 11*(1), 27-44.

Le Poul, E., Loison, C., Struyf, S., Springael, J. Y., Lannoy, V., Decobecq, M. E. & Detheux, M. (2003). Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *Journal of Biological Chemistry, 278*(28), 25481-25489.

McGeer, P. L., & McGeer, E. G. (2004). Inflammation and neurodegeneration in Parkinson's disease. Parkinsonism & related disorders, 10, S3-S7.

Le Roux, CW, & Bloom, SR (2005). Peptide YY, appetite and food intake. *Proceedings of the Nutrition Society. 64*(2), 213-216.

Lee, Alexandra, Cardel, Michelle, & Donahoo, William T. (2019). Social and Environmental Factors Influencing Obesity. In *Endotext [Internet]*: MDText. com, Inc.

Lee, Bonggi, Moon, Kyoung Mi, & Kim, Choon Young (2018). Tight junction in the intestinal epithelium: its association with diseases and regulation by phytochemicals. *Journal of immunology research. 2018*.

Lee, Do Kyung, Jang, Seok, Baek, Eun Hye, Kim, Mi Jin, Lee, Kyung Soon, Shin, Hea Soon, Chung, Myung Jun, Kim, Jin Eung, Lee, Kang Oh, & Ha, Nam Joo. (2009). Lactic acid bacteria affect serum cholesterol levels, harmful fecal enzyme activity, and fecal water content. *Lipids in Health and Disease, 8*(1), 21.

Lee, HS, Gilliland, SE, & Carter, S (2001). Amylolytic cultures of Lactobacillus acidophilus: potential probiotics to improve dietary starch utilization. *Journal of food science. 66*(2), 338-344.

Leite, Analy MO, Miguel, MAL, Peixoto, RS, Ruas-Madiedo, Patricia, Paschoalin, VMF, Mayo, B, & Delgado, Susana. (2015). Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. *Journal of dairy science, 98*(6), 3622-3632.

Leroy, Frédéric, De Vuyst, Luc (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science, & Technology. 15*(2), 67-78.

Leroy, Frédéric, Falony, Gwen, & de Vuyst, Luc. (2008). Latest developments in probiotics. In *Meat biotechnology* (pp. 217-229): Springer.

Leuschner, RG, Kenneally, PM, & Arendt, EK. (1997). Method for the rapid quantitative detection of lipolytic activity among food fermenting microorganisms. *International journal of food microbiology, 37*(2-3), 237-240.

Ley, Ruth E, Lozupone, Catherine A, Hamady, Micah, Knight, Rob, & Gordon, Jeffrey I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology, 6*(10), 776.

Li, Miao, Wang, Yi, Cui, Hongyu, Li, Yongfeng, Sun, Yuan, & Qiu, Hua-Ji (2020). Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. *Frontiers in veterinary science. 7*, 49.

Li, Xuan, Shimizu, Yuuki, Kimura, Ikuo (2017). Gut microbial metabolite short-chain fatty acids and obesity. *Bioscience of microbiota, food, & health.* 17-010.

Lim, Ye Heng, Foo, Hooi Ling, Loh, Teck Chwen, Mohamad, Rosfarizan, & Abdullah, Norhani. (2019). Comparative studies of versatile extracellular proteolytic activities of lactic acid bacteria and their potential for extracellular amino acid productions as feed supplements. *Journal of animal science and biotechnology, 10*(1), 15.

Lim, Yuen, Song, SuJin, & Song, Won. (2017). Prevalence and determinants of overweight and obesity in children and adolescents from migrant and seasonal farmworker families in the United States—a systematic review and qualitative assessment. *Nutrients, 9*(3), 188.

Löffler, Antje, Luck, Tobias, Then, Francisca S, Luck-Sikorski, Claudia, Pabst, Alexander, Kovacs, Peter, Böttcher, Yvonne, Breitfeld, Jana, Tönjes, Anke, & Horstmann, Annette. (2017). Effects of psychological eating behaviour domains on the association between socio-economic status and BMI. *Public health nutrition, 20*(15), 2706-2712.

Loos, Ruth JF, & Yeo, Giles SH. (2014). The bigger picture of FTO—the first GWAS-identified obesity gene. *Nature Reviews Endocrinology, 10*(1), 51.

Lopez-Brea, Sonia Garde, Gómez-Torres, Natalia, & Arribas, Marta Ávila (2018). Spore-forming bacteria in dairy products. *Microbiology in Dairy Processing.* 11.

Lu, Yuanyuan, Fan, Chaonan, Li, Ping, Lu, Yanfei, Chang, Xuelian, & Qi, Kemin (2016). Short chain fatty acids prevent high-fat-diet-induced obesity in mice by regulating G protein-coupled receptors and gut microbiota. *Scientific reports. 6*(1), 1-13.

Luca, Francesca, Kupfer, Sonia S, Knights, Dan, Khoruts, Alexander, & Blekhman, Ran. (2018). Functional genomics of host–microbiome interactions in humans. *Trends in Genetics, 34*(1), 30-40.

Ma, Jonathan, Rubin, Bruce K, & Voynow, Judith A (2018). Mucins, mucus, and goblet cells. *Chest. 154*(1), 169-176.

Macfarlane, Sandra, & Macfarlane, George T (2003). Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society. 62*(1), 67-72.

Mackie, Alan, Mulet-Cabero, Ana-Isabel, Torcello-Gómez, Amelia (2020). Simulating human digestion: developing our knowledge to create healthier and more sustainable foods, *Food, & Function.*

Macori, Guerrino, & Cotter, Paul D (2018). Novel insights into the microbiology of fermented dairy foods. *Current opinion in biotechnology. 49*, 172-178.

Madeeha, Ilyas Rana, Ikram, Aamer, Imran, Muhammad (2016). A preliminary insight of correlation between human fecal microbial diversity and blood lipid profile. *International journal of food sciences, & nutrition. 67*(7), 865-871.

Mahe, Maxime M, Brown, Nicole E, Poling, Holly M, & Helmrath, Michael A. (2017). In vivo model of small intestine. In *Organ Regeneration* (pp. 229-245): Springer.

Maher, Tyler, El-Chab, Alaeddine, Shafat, Amir, & Clegg, Miriam E (2019). Effect of medium-chain TAG and exercise on satiety, energy intake and energy balance. *British Journal of Nutrition. 122*(11), 1313-1320.

Mahmood, Talat, Masud, Tariq, Imran, Muhammad, Ahmed, Iftikhar, Khalid, Nauman (2013). Selection and characterization of probiotic culture of Streptococcus thermophilus from dahi. *International journal of food sciences, & nutrition. 64*(4), 494-501.

Makarova, K, Slesarev, A, Wolf, Y, Sorokin, A, Mirkin, B, Koonin, E, Pavlov, Andrey, Pavlova, N, Karamychev, V, & Polouchine, N (2006). Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences. 103*(42), 15611-15616.

Maqsood, Shabana, Hasan, Fariha, & Masud, Tariq. (2013). Characterization of lactic acid bacteria isolated from indigenous dahi samples for potential source of starter culture. *African Journal of Biotechnology, 12*(33).

Marcela, González, Olivera, Jorge A, Bermúdez, Jorge, & Reginensi, Stella M. (2016). Genetic divesity and extracelullar enzymatic activity of bacillus licheniformis strains from milk powder. *Journal of Microbiology, Biotechnology & Food Sciences, 5*(5).

Markowiak, Paulina, & Śliżewska, Katarzyna (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients. 9*(9), 1021.

Marnell, Lorraine, Mold, Carolyn, & Du Clos, Terry W (2005). C-reactive protein: ligands, receptors and role in inflammation. *Clinical immunology. 117*(2), 104-111.

Marraffini, Luciano A (2015). CRISPR-Cas immunity in prokaryotes. *526*(7571), *Nature.* 55-61.

Martinsen, Tom C, Bergh, Kåre, Waldum, Helge L (2005). Gastric juice: a barrier against infectious diseases. *Basic, pharmacology, clinical, & toxicology. 96*(2), 94-102.

Marzorati, Massimo, Vanhoecke, Barbara, De Ryck, Tine, Sadabad, Mehdi Sadaghian, Pinheiro, Iris, Possemiers, Sam, Van den Abbeele, Pieter, Derycke, Lara, Bracke, Marc, & Pieters, Jan (2014). The HMI™ module: a new tool to study the Host-Microbiota Interaction in the human gastrointestinal tract *in vitro. BMC microbiology. 14*(1), 1-14.

Masud, T, Sultana, K, & Shah, M Amin. (1991a). Incidence of lactic acid bacteria isolated from indigenous dahi. *Asian-Australasian Journal of Animal Sciences, 4*(4), 329-331.

Masud, T, Sultana, K, & Shah, M Amin (1991b). Incidence of lactic acid bacteria isolated from indigenous dahi. *Asian-Australasian Journal of Animal Sciences. 4*(4), 329-331.

Mathara, Julius Maina, Schillinger, Ulrich, Kutima, Phillip Museve, Mbugua, Samuel K, & Holzapfel, Wilhelm H (2004). Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. *International journal of food microbiology. 94*(3), 269-278.

Matouskova, Petra, Hoova, Julie, Rysavka, Petr, & Marova, Ivana. (2021). Stress effect of food matrices on viability of probiotic cells during model digestion. *Microorganisms 9*(8), 1625.

McAuliffe, Olivia. (2017). Genetics of lactic acid bacteria. In *Cheese* (pp. 227-247): Elsevier.

McCabe-Sellers, Beverly J, Staggs, Cathleen G, & Bogle, Margaret L. (2006). Tyramine in foods and monoamine oxidase inhibitor drugs: a crossroad where medicine, nutrition, pharmacy, and food industry converge. *Journal of Food Composition and Analysis, 19*, S58-S65.

McGinnis, J Michael, & Nestle, Marion. (1989). The Surgeon General's report on nutrition and health: policy implications and implementation strategies. *The American journal of clinical nutrition, 49*(1), 23-28.

McLennan, Amy K, & Ulijaszek, Stanley J. (2015). Obesity emergence in the Pacific islands: why understanding colonial history and social change is important. *Public health nutrition, 18*(8), 1499-1505.

Mehmood, Talat, Masud, Tariq, Abbass, Syed Ali, & Maqsud, Shabana (2009). Isolation and Identification of wild strains of lactic acid bacteria for yoghurt preparation from indigenous dahi. *Pakistan Journal of Nutrition. 8*(6), 866-871.

Mehrpouya-Bahrami, Pegah, Chitrala, Kumaraswamy Naidu, Ganewatta, Mitra S, Tang, Chuanbing, Murphy, E Angela, Enos, Reilly T, Velazquez, Kandy T, McCellan, Jamie, Nagarkatti, Mitzi, & Nagarkatti, Prakash (2017). Blockade of CB1 cannabinoid receptor alters gut microbiota and attenuates inflammation and diet-induced obesity. *Scientific reports. 7*(1), 1-16.

Mehta, Deepika, & Satyanarayana, Tulasi. (2016). Bacterial and archaeal α-amylases: diversity and amelioration of the desirable characteristics for industrial applications. *Frontiers in Microbiology, 7*, 1129.

Membrez, Mathieu, Blancher, Florence, Jaquet, Muriel, Bibiloni, Rodrigo, Cani, Patrice D, Burcelin, Rémy G, Corthesy, Irène, Macé, Katherine, & Chou, Chieh Jason. (2008). Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. *The FASEB Journal, 22*(7), 2416-2426.

Méndez-Salazar, Eder Orlando, Ortiz-López, María Guadalupe, Granados-Silvestre, María de los Ángeles, Palacios-González, Berenice, & Menjivar, Marta (2018). Altered gut microbiota and compositional changes in Firmicutes and Proteobacteria in Mexican undernourished and obese children. *Frontiers in microbiology*. *9*, 2494.

Menghini, Rossella, Lauro, Renato, & Federici, Massimo. (2007). *Inflammation and macrophage infiltration in adipose tissue: A link between diabetes and atherosclerosis.* Paper presented at the International Congress Series.

Meroni, Marica, Longo, Miriam, & Dongiovanni, Paola (2019). Alcohol or gut microbiota: who is the guilty?. *International journal of molecular sciences. 20*(18), 4568.

Mialon, Melissa, Swinburn, Boyd, Allender, Steven, Sacks, Gary (2017). 'Maximising shareholder value': a detailed insight into the corporate political activity of the Australian food industry. *Australian, & health, New Zealand journal of public. 41*(2), 165-171.

Michaelides, Michael, Miller, Michael L, Egervari, Gabor, Primeaux, Stefany D, Gomez, Juan L, Ellis, Randall J, Landry, Joseph A, Szutorisz, Henrietta, Hoffman, Alexander F, & Lupica, Carl R (2018). Striatal Rgs4 regulates feeding and susceptibility to diet-induced obesity. *Molecular psychiatry.* 1-12.

Miranda, Valter Paulo Neves, dos Santos Amorim, Paulo Roberto, Bastos, Ronaldo Rocha, de Faria, Eliane Rodrigues, de Castro Moreira, Maria Eliza, do Carmo Castro Franceschini, Sylvia, do Carmo Gouveia Peluzio, Maria, de Luces Fortes Ferreira, Célia Lucia, & Priore, Silvia Eloiza (2019). Abundance of gut microbiota, concentration of short-chain fatty acids, and inflammatory markers associated with elevated body fat, overweight, and obesity in female adolescents. *Mediators of inflammation. 2019*.

Mirmonsef, Paria, Zariffard, Mohammad R, Gilbert, Douglas, Makinde, Hadijat, Landay, Alan L, & Spear, Greg T (2012). Short-chain fatty acids induce pro-inflammatory cytokine production alone and in combination with toll-like receptor ligands. *American Journal of Reproductive Immunology. 67*(5), 391-400.

Mo, Zhenzhen, Huang, Shurong, Burnett, Dustin J, Rutledge, John C, & Hwang, Daniel H (2020). Endotoxin May Not Be the Major Cause of Postprandial Inflammation in Adults Who Consume a Single High-Fat or Moderately High-Fat Meal. *The Journal of Nutrition. 150*(5), 1303-1312.

Mohammadi-Sartang, M, Bellissimo, N, de Zepetnek, JO Totosy, Brett, NR, Mazloomi, SM, Fararouie, M, Bedeltavana, A, Famouri, M, Mazloom, Z (2018). The effect of daily

fortified yogurt consumption on weight loss in adults with metabolic syndrome: A 10-week randomized controlled trial. *Nutrition, Metabolism, & Diseases, Cardiovascular. 28*(6), 565-574.

Mohania, Dheeraj, Kansal, Vinod Kumar, Sagwal, Renu, Shah, Dilip (2013a). Anticarcinogenic effect of probiotic Dahi and piroxicam on DMH-induced colorectal carcinogenesis in Wistar rats. *American Journal of Cancer Therapy, & Pharmacology. 1*(1), 8-24.

Mohania, Dheeraj, Kansal, Vinod Kumar, Shah, Dilip, Nagpal, Ravinder, Kumar, Manoj, Gautam, Sanjeev Kumar, Singh, Birbal, Behare, Pradip Vishnu (2013b). Therapeutic effect of probiotic dahi on plasma, aortic, and hepatic lipid profile of hypercholesterolemic rats. *Journal of Cardiovascular pharmacology, & Therapeutics. 18*(5), 490-497.

Monda, Keri L, Gordon-Larsen, Penny, Stevens, June, & Popkin, Barry M. (2007). China's transition: the effect of rapid urbanization on adult occupational physical activity. *Social science & medicine, 64*(4), 858-870.

Monguió-Tortajada, Marta, Franquesa, Marcella, Sarrias, Maria-Rosa, Borràs, Francesc E (2018). Low doses of LPS exacerbate the inflammatory response and trigger death on TLR3-primed human monocytes. *Cell death, & disease. 9*(5), 1-14.

Mora, Diego, Dal Bello, Fabio, & Arioli, Stefania. (2018). Biological traits of lactic acid bacteria: industrial relevance and new perspectives in dairy applications. *Microbiology in Dairy Processing*, 219-232.

Moreira Júnior, Gil, Ribeiro Sobrinho, Antônio Paulino, Bambirra, Bernardo Henrique Silva, Bambirra, Felipe Henrique Silva, Carvalho, Maria Auxiliadora Roque, Farias, Luiz Macedo, Nicoli, Jacques Robert, & Moreira, Elizabeth Spangler (2011). Synergistic growth effect among bacteria recovered from root canal infections. *Brazilian Journal of Microbiology. 42*(3), 973-979.

Morrison, Douglas J, & Preston, Tom (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut microbes. 7*(3), 189-200.

Mudgal, Sreeja P, & Prajapati, Jashbhai B. (2017). Dahi—An Indian Naturally Fermented Yogurt. In *Yogurt in Health and Disease Prevention* (pp. 353-369): Elsevier.

Müller, Manfred J, Enderle, Janna, & Bosy-Westphal, Anja (2016). Changes in energy expenditure with weight gain and weight loss in humans. *Current obesity reports. 5*(4), 413-423.

Münzberg, H, Björnholm, M, Bates, SH, Myers, MG (2005). Leptin receptor action and mechanisms of leptin resistance. *Cellular, & Sciences, Molecular Life*. *62*(6), 642.

Murugan, B, & Villi, R Annal. (2009). Lipolytic activity of Bacillus species isolated from milk and dairy products. *Indian veterinary journal, 86*(1), 80-81.

Musini, A., Rao, J. P., & Giri, A. (2015). Phytochemicals of Salacia oblonga responsible for free radical scavenging and antiproliferative activity against breast cancer cell lines (MDA-MB-231). *Physiology and molecular biology of plants, 21*, 583-590.

Muscogiuri, Giovanna, Cantone, Elena, Cassarano, Sara, Tuccinardi, Dario, Barrea, Luigi, Savastano, Silvia, & Colao, Annamaria %J International journal of obesity supplements. (2019). Gut microbiota: a new path to treat obesity. *9*(1), 10-19.

Myers, Martin G, Cowley, Michael A, & Münzberg, Heike (2008). Mechanisms of leptin action and leptin resistance. *Annual Reviews of Physiology. 70*, 537-556.

Nawaz, Farah. (2008). *Genomic and Culture Based Microbiological Characterization of Fermented Milk Product Dahi and its Impact on Product Quality, Nutrition and Safety.* QUAID-I-AZAM UNIVERSITY ISLAMABAD,

Nawaz, Farah, Khan, Muhammad Nadeem, Javed, Aqib, Ahmed, Ibrar, Ali, Naeem, Ali, Muhammad Ishtiaq, Bakhtiar, Syeda Mariam, & Imran, Muhammad. (2019a). Genomic and Functional Characterization of Enterococcus mundtii QAUEM2808, Isolated From Artisanal Fermented Milk Product Dahi. *Frontiers in Microbiology, 10*.

Nawaz, Farah, Mehmood, Sadia, Ghazanfar, Shakira, Tahir, S.S., Rauf, Naseem, & Imran, Muhammad. (2016). Isolation, characterization and application of indigenous lactic acid bacteria in milk fermentation. *International journal of Biosciences, 9*(6), 415-430. doi:http://dx.doi.org/10.12692/ijb/9.6.415-430

Ngkelo, Anta, Meja, Koremu, Yeadon, Mike, Adcock, Ian, & Kirkham, Paul A (2012). LPS induced inflammatory responses in human peripheral blood mononuclear cells is mediated through NOX4 and G i α dependent PI-3kinase signalling. *Journal of inflammation. 9*(1), 1.

Noailles, Agustina, Maneu, Victoria, Campello, Laura, Lax, Pedro, & Cuenca, Nicolás. (2018). Systemic inflammation induced by lipopolysaccharide aggravates inherited retinal dystrophy. *Cell death & disease, 9*(3), 350.

Nogacka, Alicja M, Salazar, Nuria, Arboleya, Silvia, Ruas-Madiedo, Patricia, Mancabelli, Leonardo, Suarez, Adolfo, Martinez-Faedo, Ceferino, Ventura, Marco, Tochio, Takumi, & Hirano, Katsuaki (2020). In vitro evaluation of different prebiotics on the modulation of gut microbiota composition and function in morbid obese and normal-weight subjects. *International journal of molecular sciences. 21*(3), 906.

O'sullivan, Orla, O'Callaghan, John, Sangrador-Vegas, Amaia, McAuliffe, Olivia, Slattery, Lydia, Kaleta, Pawel, Callanan, Michael, Fitzgerald, Gerald F, Ross, R Paul, & Beresford, Tom (2009). Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. *BMC Microbiology*. *9*(1), 1-9.

Oddi, S, Huber, P, Duque, AL Rocha Faria, Vinderola, G, & Sivieri, Katia (2020). Breast-milk derived potential probiotics as strategy for the management of childhood obesity. *Food Research International. 137*, 109673.

oglu Gulahmadov, Saib Gurban, Batdorj, Batjargal, Dalgalarrondo, Michele, Chobert, Jean-Marc, oglu Kuliev, Akif Alekper, & Haertlé, Thomas. (2006). Characterization of bacteriocin-like inhibitory substances (BLIS) from lactic acid bacteria isolated from traditional Azerbaijani cheeses. *European Food Research and Technology, 224*(2), 229-235.

Okita, Koichi, Nishijima, Hirotaka, Murakami, Takeshi, Nagai, Tatsuya, Morita, Noriteru, Yonezawa, Kazuya, Iizuka, Kenji, Kawaguchi, Hideaki, Kitabatake, Akira (2004). Can exercise training with weight loss lower serum C-reactive protein levels? *, Arteriosclerosis, thrombosis,, & biology, vascular. 24*(10), 1868-1873.

Oliver, Stephen P, Jayarao, Bhushan M, & Almeida, Raul A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodbourne Pathogens & Disease, 2*(2), 115-129.

Oreffice, Sonia, & Quintana-Domeque, Climent. (2016). Beauty, body size and wages: evidence from a unique data set. *Economics & Human Biology, 22*, 24-34.

Ouwehand, Arthur C, Invernici, Marcos M, Furlaneto, Flávia AC, & Messora, Michel R (2018). Effectiveness of multi-strain versus single-strain probiotics: current status and recommendations for the future. *Journal of clinical gastroenterology 52*, S35-S40.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F., & Stevens, R. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res, 42*(Database issue), D206-214. doi:10.1093/nar/gkt1226

Owusu-Kwarteng, James, Akabanda, Fortune, Agyei, Dominic, & Jespersen, Lene (2020). Microbial safety of milk production and fermented dairy products in Africa. *Microorganisms. 8*(5), 752.

Paek, Jeong Ki, Lee, Seon Yeong (2018). Can Gut Microbiota Modulation Be Used as a Practical Treatment for Obesity?*, Journal of obesity, & syndrome, metabolic. 27*(2), 75.

Pakhomov, Nikolai, Baugh, John A (2020). The Role of Diet-Derived Short Chain Fatty Acids in Regulating Cardiac Pressure Overload. *American Journal of Physiology-Heart, & Physiology, Circulatory.*

Panigrahi, Sumitra, Devi, Bhanita, Swain, Krutanjali, & Priyadarshini, Priyanka (2018). Microbiology of milk: Public health aspect. *The Pharma Innovation Journal. 7*(1), 260-264.

Panzeri, Ilaria, & Pospisilik, John Andrew (2018). Epigenetic control of variation and stochasticity in metabolic disease. *Molecular metabolism. 14*, 26-38.

Papadimitriou, Konstantinos, Alegría, Ángel, Bron, Peter A, De Angelis, Maria, Gobbetti, Marco, Kleerebezem, Michiel, Lemos, José A, Linares, Daniel M, Ross, Paul, & Stanton, Catherine. (2016a). Stress physiology of lactic acid bacteria. *Microbiol. Mol. Biol. Rev., 80*(3), 837-890.

Papadimitriou, Konstantinos, Alegría, Ángel, Bron, Peter A, De Angelis, Maria, Gobbetti, Marco, Kleerebezem, Michiel, Lemos, José A, Linares, Daniel M, Ross, Paul, Stanton, Catherine (2016b). Stress physiology of lactic acid bacteria. *Microbiology, & Reviews, Molecular Biology. 80*(3), 837-890.

Pappo, Itzhak, Becovier, Herve, Berry, Elliot M, & Freund, Herbert R. (1991). Polymyxin B reduces cecal flora, TNF production and hepatic steatosis during total parenteral nutrition in the rat. *Journal of Surgical Research, 51*(2), 106-112.

Parada Venegas, Daniela, De la Fuente, Marjorie K, Landskron, Glauben, González, María Julieta, Quera, Rodrigo, Dijkstra, Gerard, Harmsen, Hermie JM, Faber, Klaas Nico, & Hermoso, Marcela A (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in immunology. 10*, 277.

Park, Gun-Seok, Park, Min Hee, Shin, Woojung, Zhao, Connie, Sheikh, Sameer, Oh, So Jung, Kim, Hyun Jung (2017). Emulating host-microbiome ecosystem of human gastrointestinal tract *in vitro*. *Stem Cell Reviews, & Reports. 13*(3), 321-334.

Pasolli, Edoardo, De Filippis, Francesca, Mauriello, Italia E, Cumbo, Fabio, Walsh, Aaron M, Leech, John, Cotter, Paul D, Segata, Nicola, & Ercolini, Danilo (2020). Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. *Nature communications. 11*(1), 1-12.

Paterson, Carley, Lacroix, Emilie, & von Ranson, Kristin M. (2019). Conceptualizing addictive-like eating: A qualitative analysis. *Appetite*, 104326.

Pereira, & Paula. (2014). Milk nutritional composition and its role in human health. *Nutrition, 30*(6), 619-627.

Pessione, Enrica, & Cirrincione, Simona. (2016). Bioactive molecules released in food by lactic acid bacteria: encrypted peptides and biogenic amines. *Frontiers in Microbiology, 7*, 876.

Philip, W, & James, T (2013). Obesity—a modern pandemic: the burden of disease. *Endocrinología y Nutrición. 60*, 3-6.

Pisoschi, Aurelia Magdalena, Pop, Aneta, Georgescu, Cecilia, Turcuş, Violeta, Olah, Neli Kinga, & Mathe, Endre. (2018). An overview of natural antimicrobials role in food. *European Journal of Medicinal Chemistry, 143*, 922-935.

Plavec, Tina Vida, & Berlec, Aleš (2020). Safety aspects of genetically modified lactic acid bacteria. *Microorganisms. 8*(2), 297.

Plaza-Diaz, Julio, Ruiz-Ojeda, Francisco Javier, Gil-Campos, Mercedes, & Gil, Angel (2019). Mechanisms of action of probiotics. *Advances in Nutrition*. *10*(suppl_1), S49-S66.

Polidori, David, Sanghvi, Arjun, Seeley, Randy J, & Hall, Kevin D (2016). How strongly does appetite counter weight loss? Quantification of the feedback control of human energy intake. *Obesity. 24*(11), 2289-2295.

Polivy, Janet, & Herman, C Peter (1999). Distress and eating: why do dieters overeat? , *International Journal of Eating Disorders. 26*(2), 153-164.

Pontonio, Erica, Dingeo, Cinzia, Gobbetti, Marco, & Rizzello, Carlo Giuseppe. (2019). Maize milling by-products: from food wastes to functional ingredients through lactic acid bacteria fermentation. *Frontiers in Microbiology, 10*, 561.

Popkin, B, Monteiro, C, & Swinburn, Boyd (2013). Overview: B ellagio C onference on P rogram and P olicy O ptions for P reventing O besity in the L ow-and M iddle-I ncome C ountries. *Obesity reviews*. *14*, 1-8.

Pradhan, Geetali, Samson, Susan L, Sun, Yuxiang (2013). Ghrelin: much more than a hunger hormone. *Current opinion in clinical nutrition, & care, metabolic. 16*(6), 619.

Puniya, M, Ravinder Kumar, M, Panwar, H, Kumar, N, & Ramneek, Anil Kumar P. (2016). Screening of lactic acid bacteria of different origin for their probiotic potential. *J Food Process Technol, 7*(545), 2.

Qi, Qibin, Chu, Audrey Y, Kang, Jae H, Jensen, Majken K, Curhan, Gary C, Pasquale, Louis R, Ridker, Paul M, Hunter, David J, Willett, Walter C, & Rimm, Eric B. (2012). Sugar-sweetened beverages and genetic risk of obesity. *New England Journal of Medicine, 367*(15), 1387-1396.

Quarta, Carmelo, Schneider, Robert, & Tschöp, Matthias H (2016). Epigenetic ON/OFF switches for obesity. *Cell. 164*(3), 341-342.

Quintanilha, Bruna Jardim, Ferreira, Ludmila Rodrigues Pinto, Ferreira, Frederico Moraes, Neto, Edécio Cunha, Sampaio, Geni Rodrigues, & Rogero, Marcelo Macedo (2020). Circulating plasma microRNAs dysregulation and metabolic endotoxemia induced by a high-fat high-saturated diet. *Clinical Nutrition. 39*(2), 554-562.

Rabot, Sylvie, Membrez, Mathieu, Bruneau, Aurélia, Gérard, Philippe, Harach, Taoufiq, Moser, Mireille, Raymond, Frederic, Mansourian, Robert, & Chou, Chieh J. (2010). Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. *The FASEB Journal, 24*(12), 4948-4959.

Radilla-Vázquez, Romina Belén, Parra-Rojas, Isela, Martínez-Hernández, Norma Edith, Márquez-Sandoval, Yolanda Fabiola, Illades-Aguiar, Berenice, & Castro-Alarcón, Natividad (2016). Gut microbiota and metabolic endotoxemia in young obese Mexican subjects. *Obesity facts. 9*(1), 1-11.

Rajilic-Stojanovic, Mirjana, Figueiredo, Ceu, Smet, Annemieke, Hansen, Richard, Kupcinskas, Juozas, Rokkas, Theo, Andersen, Leif, Machado, Jose C, Ianiro, Gianluca, Gasbarrini, Antonio (2020). Systematic review: gastric microbiota in health and disease. *Alimentary pharmacology, & therapeutics. 51*(6), 582-602.

Ramana, Kumar Panqanamala, & Pravin, Varaiya. (2015). *Stochastic systems: Estimation, identification, and adaptive control* (Vol. 75): SIAM.

Raoult, Didier, & Henrissat, Bernard. (2014). Are stool samples suitable for studying the link between gut microbiota and obesity? *European journal of epidemiology, 29*(5), 307-309.

Ray, RC, & Montet, D. (2016). Amylolytic lactic acid Bacteria: microbiology and technological interventions in food fermentations. *Fermented foods. CRC Press. Part I*, 142-159.

Raynal-Ljutovac, Ketsia, Lagriffoul, Gilles, Paccard, P, Guillet, I, & Chilliard, Yves (2008). Composition of goat and sheep milk products: An update. *Small ruminant research*. *79*(1), 57-72.

Redondo-Useros, Noemí, Gheorghe, Alina, Díaz-Prieto, Ligia E, Villavisencio, Brenda, Marcos, Ascensión, & Nova, Esther (2019). Associations of probiotic fermented milk (PFM) and yogurt consumption with *Bifidobacterium* and *Lactobacillus* components of the gut microbiota in healthy adults. *Nutrients. 11*(3), 651.

Reilly, John J, Armstrong, Julie, Dorosty, Ahmad R, Emmett, Pauline M, Ness, Andrew, Rogers, Imogen, Steer, Colin, & Sherriff, Andrea. (2005). Early life risk factors for obesity in childhood: cohort study. *BMJ, 330*(7504), 1357.

Ren, Dayong, Zhu, Jianwei, Gong, Shengjie, Liu, Hongyan, & Yu, Hansong. (2018). Antimicrobial Characteristics of Lactic Acid Bacteria Isolated from Homemade Fermented Foods. *BioMed research international, 2018*.

Requena, Teresa, Song, Ya, Peláez, Carmen, & Martínez-Cuesta, M Carmen. (2021). Modulation and metabolism of obesity-associated microbiota in a dynamic simulator of the human gut microbiota. *LWT. 141*, 110921.

Richard, Denis. (2015). Cognitive and autonomic determinants of energy homeostasis in obesity. *Nature Reviews Endocrinology, 11*(8), 489.

Rinninella, Emanuele, Raoul, Pauline, Cintoni, Marco, Franceschi, Francesco, Miggiano, Giacinto Abele Donato, Gasbarrini, Antonio, & Mele, Maria Cristina. (2019a). What is the healthy gut microbiota composition? a changing ecosystem across age, environment, diet, and diseases. *Microorganisms, 7*(1), 14.

Rinninella, Emanuele, Raoul, Pauline, Cintoni, Marco, Franceschi, Francesco, Miggiano, Giacinto Abele Donato, Gasbarrini, Antonio, & Mele, Maria Cristina (2019b). What is the healthy gut microbiota composition? a changing ecosystem across age, environment, diet, and diseases. *Microorganisms. 7*(1), 14.

Risk, NCD (2016). Factor Collaboration (NCD-RisC). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. *Lancet. 387*(10026), 1377-1396.

Rizzatti, G, Lopetuso, LR, Gibiino, G, Binda, C, & Gasbarrini, A (2017). Proteobacteria: a common factor in human diseases. *BioMed research international*. *2017*.

Roberto, Christina A, & Kawachi, Ichiro. (2015). *Behavioral economics and public health*: Oxford University Press.

Robinson, Richard K. (2005). *Dairy microbiology handbook: the microbiology of milk and milk products*: John Wiley & Sons.

Rodes, Laetitia, Coussa-Charley, Michael, Marinescu, Daniel, Paul, Arghya, Fakhoury, Marc, Abbasi, Sana, Khan, Afshan, Tomaro-Duchesneau, Catherine, Prakash, Satya (2013). Design of a novel gut bacterial adhesion model for probiotic applications. *Artificial cells, nanomedicine,, & biotechnology. 41*(2), 116-124.

Rogero, Marcelo Macedo, & Calder, Philip C (2018). Obesity, inflammation, toll-like receptor 4 and fatty acids. *Nutrients. 10*(4), 432.

Rokholm, Benjamin, Baker, Jennifer Lyn, & Sørensen, Thorkild Ingvor A. (2010). The levelling off of the obesity epidemic since the year 1999–a review of evidence and perspectives. *Obesity reviews, 11*(12), 835-846.

Romieu, Isabelle, Dossus, Laure, Barquera, Simón, Blottière, Hervé M, Franks, Paul W, Gunter, Marc, Hwalla, Nahla, Hursting, Stephen D, Leitzmann, Michael, Margetts, Barrie (2017). Energy balance and obesity: what are the main drivers? *Cancer Causes, & Control. 28*(3), 247-258.

Roncon-Albuquerque Jr, Roberto, Moreira-Rodrigues, Mónica, Faria, Bernardo, Ferreira, Andrea P, Cerqueira, Cátia, Lourenço, André P, Pestana, Manuel, von Hafe, Pedro, & Leite-Moreira, Adelino F. (2008). Attenuation of the cardiovascular and metabolic complications of obesity in CD14 knockout mice. *Life sciences, 83*(13-14), 502-510.

Rosen, Howard. (2014). Is Obesity A Disease or A Behavior Abnormality? Did the AMA Get It Right? *Missouri medicine, 111*(2), 104.

Rosenbaum, Michael, Knight, Rob, Leibel, Rudolph L (2015). The gut microbiota in human energy homeostasis and obesity. *Trends in Endocrinology, & Metabolism. 26*(9), 493-501.

Rosenbaum, Michael, & Leibel, Rudolph L (2014). 20 years of leptin: role of leptin in energy homeostasis in humans. *Journal of Endocrinology. 223*(1), T83-T96.

Rotimi, Charles N, Cooper, Richard S, Ataman, Susan L, Osotimehin, Babatunde, Kadiri, Solomon, Muna, Walinjom, Kingue, Samuel, Fraser, Henry, & McGee, Daniel. (1995). Distribution of anthropometric variables and the prevalence of obesity in populations of west African origin: the International Collaborative Study on Hypertension in Blacks (ICSHIB). *Obesity research, 3*(S2), 95s-105s.

Rowland, Ian, Gibson, Glenn, Heinken, Almut, Scott, Karen, Swann, Jonathan, Thiele, Ines, & Tuohy, Kieran. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *European journal of nutrition, 57*(1), 1-24.

Sadeghirad, B, Duhaney, T, Motaghipisheh, S, Campbell, NRC, & Johnston, BC. (2016). Influence of unhealthy food and beverage marketing on children's dietary intake and preference: a systematic review and meta-analysis of randomized trials. *Obesity reviews, 17*(10), 945-959.

Saito, Toshiyuki, Hayashida, Hideaki, & Furugen, Reiko. (2007). Comment on: Cani et al.(2007) Metabolic endotoxemia initiates obesity and insulin resistance: Diabetes 56: 1761–1772. *Diabetes, 56*(12), e20-e20.

Samad, Mohd Yusof A, Razak, C Nyonya A, Salleh, Abu Bakar, Yunus, WM Zin Wan, Ampon, Kamaruzaman, & Basri, Mahiran. (1989). A plate assay for primary screening of lipase activity. *Journal of microbiological methods, 9*(1), 51-56.

Sams, Laura, Paume, Julie, Giallo, Jacqueline, Carrière, Frédéric (2016). Relevant pH and lipase for in vitro models of gastric digestion. *Food, & function. 7*(1), 30-45.

Sanchis-Chordà, Jesús, Del Pulgar, Eva M Gómez, Carrasco-Luna, Joaquín, Benítez-Páez, Alfonso, Sanz, Yolanda, & Codoñer-Franch, Pilar (2019). *Bifidobacterium pseudocatenulatum* CECT 7765 supplementation improves inflammatory status in insulin-resistant obese children. *European journal of nutrition. 58*(7), 2789-2800.

Sanders, Mary Ellen (2008). Probiotics: definition, sources, selection, and uses. *Clinical infectious diseases. 46*(Supplement_2), S58-S61.

Sanders, ME, Merenstein, D, Merrifield, CA, & Hutkins, R (2018). Probiotics for human use. *Nutrition bulletin. 43*(3), 212-225.

Sanz, Yolanda, Marta Olivares, Ángela Moya-Pérez, and Carlo Agostoni. "Understanding the role of gut microbiome in metabolic disease risk." *Pediatric research 77,* no. 1 (2015): 236-244.

Trivedi, D. K., Sinclair, E., Xu, Y., Sarkar, D., Walton-Doyle, C., Liscio, C., ... & Barran, P. (2019). Discovery of volatile biomarkers of Parkinson's disease from sebum. ACS central science, 5(4), 599-606.

Satokari, Reetta. (2019). Modulation of gut microbiota for health by current and next-generation probiotics. In: Multidisciplinary Digital Publishing Institute.

Scarborough, Peter, Burg, Melanie R, Foster, Charlie, Swinburn, Boyd, Sacks, Gary, Rayner, Mike, Webster, Premila, & Allender, Steven. (2011). Increased energy intake entirely accounts for increase in body weight in women but not in men in the UK between 1986 and 2000. *British journal of nutrition, 105*(9), 1399-1404.

Schneider, Hannah, Pelaseyed, Thaher, Svensson, Frida, & Johansson, Malin EV (2018). Study of mucin turnover in the small intestine by in vivo labeling. *Scientific reports. 8*(1), 1-11.

Schrezenmeir, Jürgen, & de Vrese, Michael (2001). Probiotics, prebiotics, and synbiotics—approaching a definition. *The American journal of clinical nutrition. 73*(2), 361s-364s.

Schulz, Leslie O, Bennett, Peter H, Ravussin, Eric, Kidd, Judith R, Kidd, Kenneth K, Esparza, Julian, & Valencia, Mauro E. (2006). Effects of traditional and western environments on prevalence of type 2 diabetes in Pima Indians in Mexico and the US. *Diabetes care, 29*(8), 1866-1871.

Schulze, Matthias B, Manson, JoAnn E, Ludwig, David S, Colditz, Graham A, Stampfer, Meir J, Willett, Walter C, & Hu, Frank B. (2004). Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. *Jama, 292*(8), 927-934.

Sekirov, Inna, Russell, Shannon L, Antunes, L Caetano M, & Finlay, B Brett (2010). Gut microbiota in health and disease. *Physiological reviews*.

Seong, Chi Nam, Kang, Joo Won, Lee, Ji Hee, Seo, So Yeon, Woo, Jung Jae, Park, Chul, Bae, Kyung Sook, & Kim, Mi Sun (2018). Taxonomic hierarchy of the phylum Firmicutes and novel Firmicutes species originated from various environments in Korea. *Journal of Microbiology. 56*(1), 1-10.

Sergeev, Igor N, Aljutaily, Thamer, Walton, Gemma, & Huarte, Eduardo (2020). Effects of synbiotic supplement on human gut microbiota, body composition and weight loss in obesity. *Nutrients. 12*(1), 222.

Shangpliang, HNJ, Sharma, Sharmila, Rai, Ranjita, & Tamang, Jyoti P (2017). Some technological properties of lactic acid bacteria isolated from Dahi and Datshi, naturally fermented milk products of Bhutan. *Frontiers in Microbiology. 8*, 116.

Shin, Na-Ri, Whon, Tae Woong, & Bae, Jin-Woo (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends in biotechnology. 33*(9), 496-503.

Shokryazdan, Parisa, Faseleh Jahromi, Mohammad, Liang, Juan Boo, & Ho, Yin Wan (2017). Probiotics: from isolation to application. *Journal of the American College of Nutrition. 36*(8), 666-676.

Sides-Moore, Lauren, & Tochkov, Karin. (2011). The thinner the better? Competitiveness, depression and body image among college student women. *College Student Journal, 45*(2), 439-449.

Simonová, M, Sirotek, K, Marounek, M, & Lauková, A. (2008). Lipolytic activity of potential probiotic Enterococci and additive Staphylococci. *Acta Veterinaria Brno, 77*(4), 575-580.

Snowdon, Wendy, & Thow, Anne Marie. (2013). Trade policy and obesity prevention: challenges and innovation in the P acific I slands. *Obesity reviews, 14*, 150-158.

Soltani, M, Shenavar Masouleh, A, Ahmadi, M, Pourkazemi, M, & Taherimirghaed, A. (2016). Antibacterial activity, antibiotic susceptibility and probiotic use of lactic acid bacteria (LAB) in Persian sturgeon (Acipenser persicus). *Iranian Journal of Aquatic Animal Health, 2*(1), 54-65.

Somashekaraiah, Rakesh, Shruthi, B, Deepthi, BV, & Sreenivasa, MY (2019). Probiotic properties of lactic acid bacteria isolated from neera: a naturally fermenting coconut palm nectar. *Frontiers in microbiology. 10*, 1382.

Stangl, Anne L, Earnshaw, Valerie A, Logie, Carmen H, van Brakel, Wim, Simbayi, Leickness C, Barré, Iman, & Dovidio, John F. (2019). The Health Stigma and Discrimination Framework: a global, crosscutting framework to inform research, intervention development, and policy on health-related stigmas. *BMC medicine, 17*(1), 31.

Steinert, Robert E, Feinle-Bisset, Christine, Asarian, Lori, Horowitz, Michael, Beglinger, Christoph, & Geary, Nori (2017). Ghrelin, CCK, GLP-1, and PYY (3–36): secretory controls and physiological roles in eating and glycemia in health, obesity, and after RYGB. *Physiological reviews*. *97*(1), 411-463.

Stojanov, Spase, Berlec, Aleš, & Štrukelj, Borut (2020). The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. *Microorganisms. 8*(11), 1715.

Suganami, Takayoshi, Tanimoto-Koyama, Kanami, Nishida, Junko, Itoh, Michiko, Yuan, Xunmei, Mizuarai, Shinji, Kotani, Hidehito, Yamaoka, Shoji, Miyake, Kensuke, & Aoe, Seiichiro. (2007). Role of the Toll-like receptor 4/NF-κB pathway in saturated fatty acid–induced inflammatory changes in the interaction between adipocytes and macrophages. *Arteriosclerosis, thrombosis, and vascular biology, 27*(1), 84-91.

Suhonen, Anniina. (2019). Antibiotic Susceptibility of Lactic Acid Bacteria.

Swinburn, Boyd A, Sacks, Gary, Hall, Kevin D, McPherson, Klim, Finegood, Diane T, Moodie, Marjory L, & Gortmaker, Steven L. (2011). The global obesity pandemic: shaped by global drivers and local environments. *The Lancet, 378*(9793), 804-814.

Swinburn, Boyd A, Sacks, Gary, Lo, Sing Kai, Westerterp, Klaas R, Rush, Elaine C, Rosenbaum, Michael, Luke, Amy, Schoeller, Dale A, DeLany, James P, & Butte, Nancy F. (2009). Estimating the changes in energy flux that characterize the rise in obesity prevalence. *The American journal of clinical nutrition, 89*(6), 1723-1728.

Szulińska, Monika, Łoniewski, Igor, Van Hemert, Saskia, Sobieska, Magdalena, & Bogdański, Paweł (2018). Dose-dependent effects of multispecies probiotic supplementation on the lipopolysaccharide (LPS) level and cardiometabolic profile in obese postmenopausal women: A 12-week randomized clinical trial. *Nutrients*. *10*(6), 773.

Szutowska, Julia (2020). Functional properties of lactic acid bacteria in fermented fruit and vegetable juices: A systematic literature review. *European Food Research, & Technology. 246*(3), 357-372.

Takagi, Risa, Sasaki, Kengo, Sasaki, Daisuke, Fukuda, Itsuko, Tanaka, Kosei, Yoshida, Ken-ichi, Kondo, Akihiko, & Osawa, Ro (2016). A single-batch fermentation system to simulate human colonic microbiota for high-throughput evaluation of prebiotics. *PLoS One. 11*(8), e0160533.

Tanaka, H, Doesburg, K, Iwasaki, T, & Mierau, I (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *Journal of dairy science. 82*(12), 2530-2535.

Tappia, Paramjit S, & Defries, Danielle. (2020). Prevalence, Consequences, Causes and Management of Obesity. In *Pathophysiology of Obesity-Induced Health Complications* (pp. 3-22): Springer.

Teixeira, Pedro J, & Marques, Marta M. (2017). Health behavior change for obesity management. *Obesity facts, 10*(6), 666-673.

Thursby, Elizabeth, & Juge, Nathalie (2017). Introduction to the human gut microbiota. *Biochemical Journal. 474*(11), 1823-1836.

Topping, David L (1996). Short-chain fatty acids produced by intestinal bacteria. *Asia Pacific journal of clinical nutrition. 5*, 15-19.

Tsai, Cheng-Chih, Lin, Pei-Pei, & Hsieh, You-Miin. (2008). Three Lactobacillus strains from healthy infant stool inhibit enterotoxigenic Escherichia coli grown in vitro. *Anaerobe, 14*(2), 61-67.

Tsitko, Irina, Wiik-Miettinen, Fanny, Mattila, Outi, Rosa-Sibakov, Natalia, Seppänen-Laakso, Tuulikki, Maukonen, Johanna, Nordlund, Emilia, & Saarela, Maria (2019). A small in vitro fermentation model for screening the gut microbiota effects of different fiber preparations. International journal of molecular sciences. *20*(8), 1925.

Turnbaugh, Peter J, Ley, Ruth E, Mahowald, Michael A, Magrini, Vincent, Mardis, Elaine R, & Gordon, Jeffrey I (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. *444*(7122), 1027-1031.

Upadhyay, Jagriti, Farr, Olivia, Perakakis, Nikolaos, Ghaly, Wael, & Mantzoros, Christos (2018). Obesity as a disease. *Medical Clinics. 102*(1), 13-33.

Uriot, Ophélie, Kebouchi, Mounira, Lorson, Emilie, Galia, Wessam, Denis, Sylvain, Chalancon, Sandrine, Hafeez, Zeeshan, Roux, Emeline, Genay, Magali, & Blanquet-Diot, Stéphanie (2021). Identification of Streptococcus thermophilus Genes Specifically Expressed under Simulated Human Digestive Conditions Using R-IVET Technology. *Microorganisms*. *9*(6), 1113.

Vancamelbeke, Maaike, Vermeire, Séverine (2017). The intestinal barrier: a fundamental role in health and disease. *Expert review of gastroenterology, & hepatology. 11*(9), 821-834.

Vasiljevic, Todor, & Shah, Nagendra P. (2008). Probiotics—from Metchnikoff to bioactives. *International Dairy Journal, 18*(7), 714-728.

Wang, Gang, Han, Tao, Wang, ShiJia, Chen, Min, Sun, Yueming, & Fu, Zan (2018). Peroxisome Proliferator-Activated Receptor-Prevents Cholesterol Gallstone Formation in C57bl Mice by Regulating Bile Acid Synthesis and Enterohepatic Circulation. *BioMed research international. 2018*.

Wang, Miaomiao, Wichienchot, Santad, He, Xiaowei, Fu, Xiong, Huang, Qiang, Zhang, Bin (2019). *In vitro* colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. *Trends in food science, & technology. 88*, 1-9.

Wangensteen, T, Undlien, D, Tonstad, S, & Retterstøl, L (2005). Genetic causes of obesity. *Tidsskrift for den Norske laegeforening: tidsskrift for praktisk medicin, ny raekke. 125*(22), 3090-3093.

Wansink, Brian. (2011). Mindless eating: environmental contributors to obesity.

Weaver, J. A., Beverly, B. E., Keshava, N., Mudipalli, A., Arzuaga, X., Cai, C.,& Yost, E. E. (2020). Hazards of diethyl phthalate (DEP) exposure: A systematic review of animal toxicology studies. *Environment international, 145, 105* 848.

Weihrauch-Blüher, Susann, Schwarz, Peter, & Klusmann, Jan-Henning (2019). Childhood obesity: increased risk for cardiometabolic disease and cancer in adulthood. *Metabolism. 92*, 147-152.

Weiss, Ram, & Gepstein, Vardit. (2019). Obesity as the main risk factor for metabolic syndrome in children. *Frontiers in endocrinology, 10*, 568.

Wiciński, Michał, Gębalski, Jakub, Gołębiewski, Jakub, & Malinowski, Bartosz (2020). Probiotics for the Treatment of Overweight and Obesity in Humans—A Review of Clinical Trials. *Microorganisms. 8*(8), 1148.

Widyastuti, Yantyati, & Febrisiantosa, Andi. (2014). The role of lactic acid bacteria in milk fermentation. *Food and Nutrition Sciences, 5*(4), 720-726.

Wiese, Maria, Khakimov, Bekzod, Nielsen, Sebastian, Sørensen, Helena, van den Berg, Frans, & Nielsen, Dennis Sandris (2018). CoMiniGut—a small volume in vitro colon model for the screening of gut microbial fermentation processes. *PeerJ. 6*, e4268.

Williams, CF, Walton, GE, Jiang, L, Plummer, S, Garaiova, I, Gibson, Glenn R (2015). Comparative analysis of intestinal tract models. *Annual review of food science, & technology. 6*, 329-350.

World Health Organization (2013). Global action plan for the prevention and control of noncommunicable diseases 2013-2020.

World Health Organization Geneva: World Health Organization. (2018). Draft thirteenth general programme of work, 2019-2023.

Wostmann, Bernard S, Larkin, C, Moriarty, A, & Bruckner-Kardoss, E (1983). Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. *Laboratory animal science. 33*(1), 46-50.

Wu, Chongde, Huang, Jun, & Zhou, Rongqing (2017). Genomics of lactic acid bacteria: Current status and potential applications. *Critical Reviews in Microbiology. 43*(4), 393-404.

Yadav, Hariom, Jain, Shalini, & Sinha, PR (2007). Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition. 23*(1), 62-68.

Yan, Fang, & Polk, D Brent (2020). Probiotics and Probiotic-Derived Functional Factors—Mechanistic Insights Into Applications for Intestinal Homeostasis. *Frontiers in immunology. 11*, 1428.

Yang, Seo Jin, Kim, Kee-Tae, Kim, Tae Young, & Paik, Hyun-Dong (2020). Probiotic Properties and Antioxidant Activities of Pediococcus pentosaceus SC28 and Levilactobacillus brevis KU15151 in Fermented Black Gamju. *Foods. 9*(9), 1154.

Yanovski, Jack A. (2018). Obesity: Trends in underweight and obesity—scale of the problem. *Nature Reviews Endocrinology, 14*(1), 5.

Yao, Mingfei, Xie, Jiaojiao, Du, Hengjun, McClements, David Julian, Xiao, Hang, Li, Lanjuan (2020). Progress in microencapsulation of probiotics: A review. *Comprehensive Reviews in Food Science, & Safety, Food*. *19*(2), 857-874.

Younus, Shahid, Masud, Tariq, & Aziz, Tariq. (2002). Quality evaluation of market yoghurt/dahi. *Pakistan Journal of Nutrition, 1*(5), 226-230.

Zarour, Kenza, Prieto, Alicia, Pérez-Ramos, Adrián, Kihal, Mebrouk, & López, Paloma (2018). Analysis of technological and probiotic properties of Algerian L. mesenteroides strains isolated from dairy and non-dairy products. *Journal of functional foods. 49*, 351-361.

Zhang, Bowei, Sun, Wenlong, Yu, Na, Sun, Jin, Yu, Xiaoxia, Li, Xia, Xing, Yan, Yan, Dong, Ding, Qianzhi, & Xiu, Zhilong (2018). Anti-diabetic effect of baicalein is associated with the modulation of gut microbiota in streptozotocin and high-fat-diet induced diabetic rats. *Journal of Functional Foods. 46*, 256-267.

Zhou, Y, Drouin, P, & Lafreniere, C. (2016). Effect of temperature (5–25 C) on epiphytic lactic acid bacteria populations and fermentation of whole-plant corn silage. *Journal of Applied Microbiology, 121*(3), 657-671.

Zhou, You, Liang, Yongjie, Lynch, Karlene H, Dennis, Jonathan J, & Wishart, David S (2011). PHAST: a fast phage search tool. *Nucleic acids research*. *39*(suppl_2), W347-W352.

Zommiti, Mohamed, Feuilloley, Marc GJ, & Connil, Nathalie (2020). Update of Probiotics in Human World: A Nonstop Source of Benefactions till the End of Time. *Microorganisms. 8*(12), 1907.

Zoumpopoulou, Georgia, Tzouvanou, Alexandra, Mavrogonatou, Eleni, Alexandraki, Voula, Georgalaki, Marina, Anastasiou, Rania, Papadelli, Marina, Manolopoulou, Eugenia, Kazou, Maria, & Kletsas, Dimitris. (2018). Probiotic features of lactic acid bacteria isolated from a diverse pool of traditional Greek dairy products regarding specific strain-host interactions. *Probiotics and antimicrobial proteins, 10*(2), 313-322.

Zuo, Zhi-han, Shang, Bi-jiao, Shao, Ying-chun, Li, Wen-yue, Sun, Jin-sheng (2019). Screening of intestinal probiotics and the effects of feeding probiotics on the growth, immune, digestive enzyme activity and intestinal flora of Litopenaeus vannamei. *Fish, & immunology, shellfish*. *86*, 160-168.

Appendixes

Appendixes

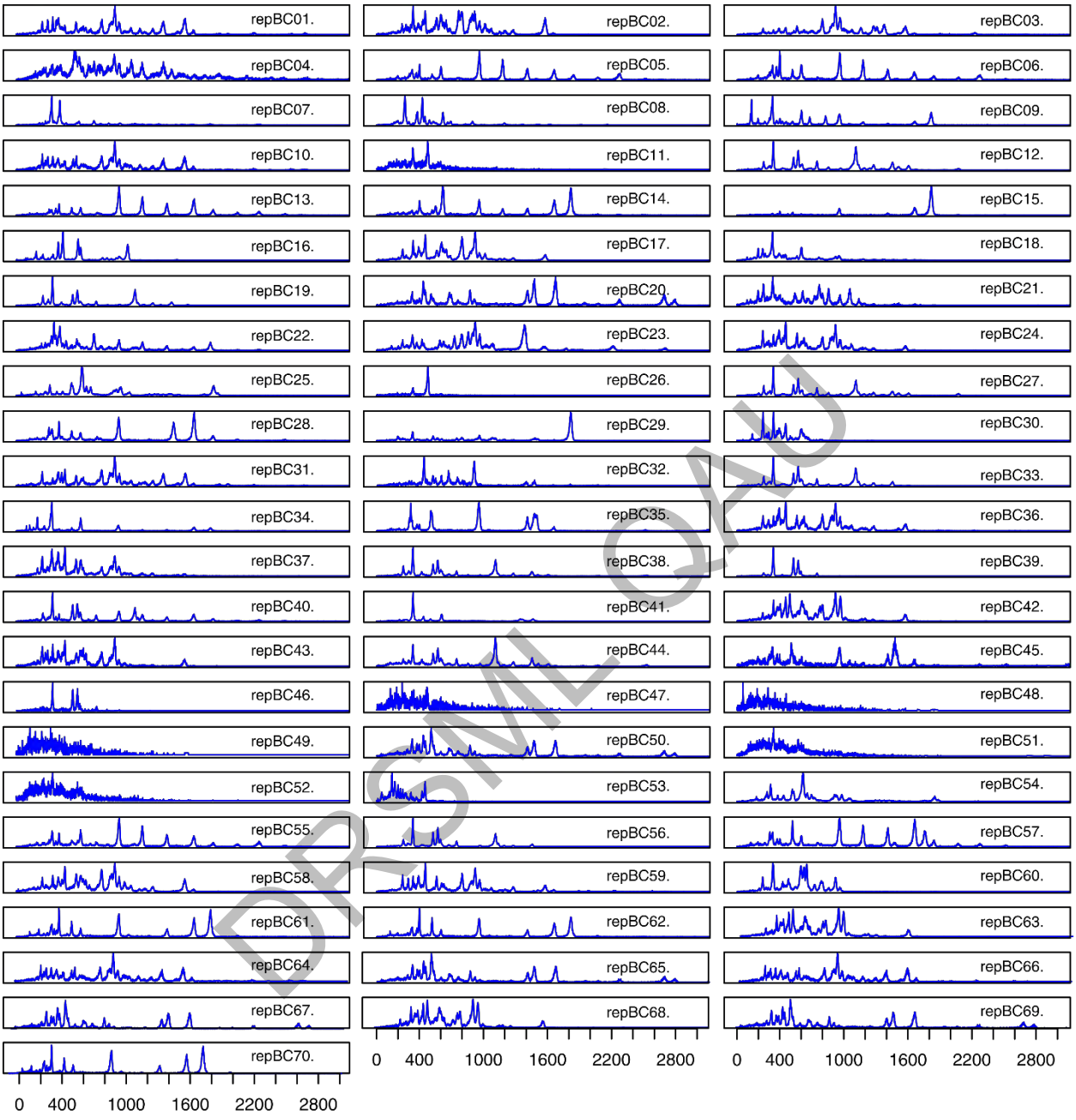

Figure 1: Least clustered peaks obtained during the Rep-seq identification method for all the seventy bacterial strains isolated from traditional fermented milk product Dahi.

Table 1: Type of fermentation and sugars that are fermented by the probiotic candidates isolated from traditional fermented milk product (Dahi).

LAB Isolates	Homo/Hetero	Sugars									
		Lactose	Glucose	Galactose	Sucrose	Dextrose	Mannose	Xylose	Fructose	Maltose	Sorbitol
L. lactis QAULLNA8	Homo	+	+	-	-	+	-	+	-	-	-
E. faecium QAUEFNA13	Hetero	+	+	+	+	+	+	+	+	+	+
E. faecium QAUEFNA17	Homo	+	+	+	+	+	+	+	+	+	+
E. faecium QAUEFNN2	Hetero	+	+	+	+	+	+	+	+	+	+
E. faecium QAUEFNN4	Hetero	+	+	+	+	+	+	+	-	+	+
E. lactis QAUELNN14	Hetero	+	+	+	+	+	+	+	+	+	+
E. faecium QAUEFNS1	Hetero	+	+	+	+	+	+	+	+	+	+
L. rhamnosus QAULRN2	Homo	+	+	+	+	+	+	+	+	+	+
L. paracasae QAULPN3	Homo	+	+	+	+	+	-	+	+	+	+
L. delbrueckii QAULDN14	Homo	+	+	+	+	+	+	-	+	+	+
L. reutori QAULRN15	Homo	+	+	+	+	+	-	+	+	+	+
L. reutori QAULRN18	Homo	+	+	+	-	+	-	+	+	+	+
L. fermentum QAULFN21	Homo	+	+	+	+	+	+	+	+	+	+
L. acidophilus QAULAN51	Homo	+	+	+	-	+	-	+	+	+	+
L. fermentum QAULFN53	Homo	+	+	+	+	+	+	+	+	+	+
L. fermentum QAULFN54	Homo	+	+	+	+	+	+	+	+	+	+
L. fermentum QAULFN55	Homo	+	+	+	+	+	+	+	+	+	+
L. fermentum QAULFN56	Homo	+	+	-	-	+	-	+	+	+	+
L. delbrueckii QAULDN61	Homo	+	+	+	+	+	+	+	+	+	+
L. fermentum QAULFN62	Homo	+	+	+	+	+	+	+	+	+	+
S. thermophilus QAUSTN63	Homo	+	+	+	+	+	+	+	+	+	+
L. fermentum QAULFN64	Homo	+	+	-	+	+	-	+	+	+	+

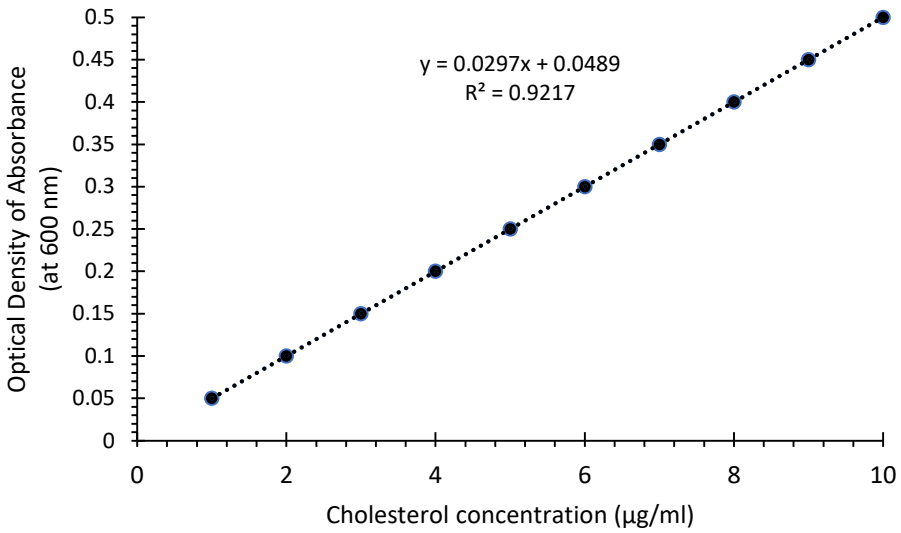

Figure 2: The standard curve used evaluation of *in vitro* cholesterol assimilation by probiotic bacteria isolated from fermented milk product Dahi.

Table 2: Detail of Microbiota used in the Ileum phase of TSI model.

S. No	Species	Strain source	Origin
1	*Escherichia coli*	DSM 1058	Human origin
2	*Streptococcus salivarius*	DSM 20560	Blood
3	*Streptococcus luteinensis*	DSM 15350	Human origin
4	*Enterococcus faecalis*	DSM 20478	Human origin
5	*Bacteroides fragilis*	DSM 2151	Appendix abscess
6	*Veillonella parvula*	DSM 2008	Human intestine
7	*Flavonifractor plautii*	DSM 6740	Human faeces