Study of the Impact of Energy Restriction (Ramadan Fasting) on Human Female Physiology and Gut Microbiome

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بسم الله الرحمن الرحيم

Certificate

This thesis submitted by ***Sidra Irshad Khan*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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DEDICATION

Dedicated to

My Beloved Parents, Siblings

and Teachers

SIDRA IRSHAD KHAN

DECLARATION

I hereby declare that the thesis entitled **"Evaluation of the Impact of Energy Restriction (Ramadan Fasting) on Human Female Physiology and Gut Microbiome"** is my own work. The material and information contained in this thesis is a record of my original work. I have not previously presented any part of this work elsewhere for any other degree.

SIDRA IRSHAD KHAN

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ABSTRACT

Over the past few decades, there is an escalating debate on impact of calorie restriction for weight loss strategy, disease management and prevention. Henceforth, keeping its importance in contemporary era, current study aims to evaluate the impact and association of calorie restriction on healthy female physiology, gut microbiota and disease prevention. The human gut is occupied by a diverse array of microorganisms which play significant role in human heath, physiology and disease prevention. Evidence suggests that dysbiosis in the gut microbiota is related to obesity and could contribute to development of obesity related metabolic diseases and many other chronic diseases. Therefore, these disorders can be cured by positive modulation of gut microbiota. Fourteen well healthy females were recruited for the study: their blood and faecal samples were collected before Ramadan and at the end of the Ramadan. Blood parameters and anthropometric values were noted to investigate the impact of Ramadan on human physiology. Effect on gut microbiology was assessed by both culture dependent and independent techniques (Metagenomics) using *Illumina Miseq* platform. Current study revealed that Ramadan fasting reduces the body weight and improves blood lipid profile such as increased "high density lipoprotein", decreased "Total Cholesterol", "Total Triglycerides", "Low Density Lipoproteins" and "very Low Density Lipoproteins". Ramadan fasting decreased culturable aerobic bacterial count and increased fungal count. It was also found that the gut metagenome is altered considerably after Ramadan. The human faecal metagenome exhibited significant changes as in decreased overall bacterial population, increased bacterial diversity (alpha diversity), and promoted evenness within bacterial population at species level. Pathogenic bacteria were decreased in F7 while increased in F23 after the study. Anti-inflammatory bacteria *Lactobacillus* and *Bifidobacterium* were favorably increased. It is conclusive from the study that Gut Microbiota of Pakistani population is dominated by Bacteriodetes and Firmicutes. Calorie Restriction has direct impact on gut microbiota in term of diversity (richness, evenness). Also, diet has strong influence on gut microbiota.

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List of Acronyms

ADF	Alternate Day Fasting
BHIA	Brain Heart Infusion Agar
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
BSH	Bile Salt Hydrolase
CAD	Coronary Artery Diseases
CFU	Colony Forming Unit
CR	Calorie Restriction
CVDs	Cardiovascular Diseases
DNA	Deoxyribonucleic Acid
GABA	Gamma Amino Butyric Acid
GIT	Gastrointestinal Tract
H_2	Hydrogen Gas
H_2O_2	Hydrogen Peroxide
LAB	Lactic Acid Bacteria
LDL	Low Density Lipoproteins
LPS	Lipopolysaccharide
MRSA	De Man, Rogosa and Sharpe Agar

MyD88	Myeloid Differentiation Primary Response Gene 88
NAFLD	Non-Alcoholic Fatty Liver Disease
NCBI	National Center for Biotechnology Information
NOD	Non-Obese Diabetic
OGA	Oxytetracyclin Glucose Agar
RNA	Ribonucleic Acid
RPM	Revolution Per Minute
SCFAs	Short Chain Fatty Acids
TCR	Total Calorie Restriction
TLRs	Toll Like Receptors
TMA	Trimethylamine
TMAO	Trimethylamine N-Oxide
WHO	World Health Organization

Chapter 1

Introduction

1 Introduction

Human physiology and physiological variations not merely manifest our genes and epigenomes but it is also a reflection of the genes and genetic variations existing in our resident microbiota. Substantial and keen interest on gut microbiota have been depicted in the recent years among scientific communities (Jandhyala *et al.*, 2015). This microbiota is present everywhere in and at human body. Surprisingly, microbiomes constitute as a minimum 100 times more genes than human genomes (Qin *et al.*, 2010). These MO co-exist in a commensal or symbiotic relationship with host and are generally harmless (Lozupone *et al.*, 2013). Beside bacteria many other microbes including fungi, archaea, viruses, and protozoans are also the part of human gut microbiota. In humans the most densely populated anatomical region is colon which constitutes the most diverse species and number of bacteria (10^{11}-10^{12} CFUs/ml) as compared to other parts (Zhao *et al.*, 2013) . Other proximal sites in the gut, including the stomach and duodenum are also inhabited by microbes but in smaller numbers (<10^3 to 10^4 CFUs/ml) (Zhao *et al.*, 2013). The human gut microflora mainly constitutes bacteria from two major phyla, Bacteriodetes & Firmicutes, with other less abundant phyla such as Actinobacteria, Proteobacteria & Verrucomicrobia (Ley *et al.*, 2005). *Methanogenic archaea* from the phyla Euryarchaeota are also present (Qin *et al.*, 2010). There are 6 divisions of bacteria present in the gut microflora which are Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia, Firmicutes and Bacteroidetes with about 90% of the bacteria belong to either the *Firmicutes* or Bacteroidetes (Cho & Blaser, 2012).

A dynamic balance occurs between bacterial communities and host physiology with many factors influencing the initial acquisition, succession, and finally till stabilization of the gut ecosystem. Colonization process starts at birth and continues till stabilization with many factors such as method of delivery, age, diet and host genetics etc. effecting the process of colonization (Dominguez *et al.*, 2010).

The functions of human gut microbiota include breakdown of indigestible dietary components by producing SCFs. These SCFA could then act as a source of energy for colonocytes along many other effects e.g. increasing satiety, decreasing inflammation, decreasing carcinogenesis in the colon and enhancing gut barrier function (Zhao *et al.*, 2013). Other functions of gut microbiota include nutrient and drug metabolism, proliferation of epithelial cells, immunological and barrier function against enteric pathogens and the synthesis of various vitamins (Marteyn *et al.*, 2010).

However, despite performing many essential function s, a state of imbalance (dysbiosis) can occur in gut, that leads to many diseases as ranged from luminal diseases (IBD and IBS) to metabolic diseases (obesity and diabetes) and allergic diseases to neurological disorders (Larsen *et al.*, 2010 ; Spencer *et al.*, 2011).

Several culture base techniques are used to study gut microbiota. However, only 10-20% gut microbiota has been isolated, thus limiting the effective use of this technique (Stewart, 2012). The reasons are: many MO are recalcitrant to growth in laboratory, it is time consuming also majority of gut microorganisms are anaerobic (Lagier *et al.*, 2015) and it is difficult to study the culture characteristics of some colonies on petri plate (Rajilić-Stojanović & de Vos, 2014).

To overcome these discrepancies, various efficient techniques have been developed to study the different underlying aspects of human gut microbiota. With the availability of different bioinformatics tools and high throughput NGS sequencing technologies (16S rRNA sequencing) which can generate many more sequences at shorter read length. This has brought about a revolution in addressing a questions concerning bacterial community structure and diversity (Jandhyala *et al.*, 2015).

Metagenomics is a technique and a process used to characterize the metagenome (all DNA from environment) (Gill *et al.*, 2006). Metagenome is the collection of genes and genomes from the species of the microflora obtained by shotgun sequencing of DNA extracted from a sample (metagenomics) followed by assembly or mapping to a reference database followed by annotation. Sequence based metagenomic analyses have begun to exposed the core metabolic functions of the human gut microflora and determine the extant properties of this community that impact on human health (Eckburg *et al.*, 2005).

Metagenomics for the Human Intestinal Tract abbreviated as MetaHIT and the Human Microbiome Project consortium are two main projects that have characterized the healthy human gut microflora (Qin *et al.*, 2010). The core objective of these projects is to find if there is present any identifiable 'core microbiome'. Though all the eighteen gut microbiomes surveyed depicted a great level of beta-diversity regarding the relative abundance of bacterial phyla. These studies concluded that different individuals may share related species. Despite the differences in species composition between individuals the metagenomics profiles were similar. This illustrates that many related species of gut bacteria are functionally redundant.

meanwhile different but related taxa can carry out similar functions in different individuals (Consortium, 2012).

The study of composition and the factors driving changes in gut microbiota is important because many metabolic, immunological and neurological disorders have been associated with gut dysbiosis. This dysbiosis, whether at the level of the phylum or more specific taxon level, aids in the advances of metabolic diseases (obesity and diabetes etc.). Overweight and obesity are alarming problems of the world, effecting both the developed and under developed countries. It is a risk factor for the development of chronic diseases like type 2 diabetes, hypertension, CVD, CAD, heart stroke and inflammatory disorders etc. (Kopelman, 2007) . In year 2014, percentage of obesity in adults in US was 35% (Ogden *et al.*, 2015), while in India it was 28% (Ng *et al.*, 2014) Pakistan is ranked 9th among 188 obese countries (Tribne, 2014). According to WHO 38% of Pakistani females and 28% males are obese.

It is known that alterations in the human gut microflora are linked with obesity but early reports are somehow inconsistent in the specific correlations. The starring role of the gut microflora in obesity related diseases have been studied using 16S rRNA sequencing and quantitative PCR, respectively (Koren *et al.*, 2011; Larsen *et al.*, 2010). However, 16S rRNA sequencing can reveal differences in the taxonomic makeup of the microflora but to discern the functional capacity of the genomes of the microorganisms in the gut, metagenomic sequencing is required.

To overcome obesity and to maintain energy level fasting is carried out. Fasting is an act of abstaining every kind of food for approximately 12 hours depending on sunrise and sunset time of that region. Thus, in a general sense it's a processing of restricting energy intake. Based on energy intake level fasting is of two main types. First is "Caloric Restriction (CR)" which is reducing the utilization of food and depriving of certain nutrients thus resulting in malnutrition. Second one is "Total Caloric Desistance (TCD)" similar to intermittent fasting, (ADF), routine periodic fasting (Rizza, Veronese, & Fontana, 2014). Various religious followers fast according to their own beliefs and rituals including Muslims, who fast from sunrise (Sehr) to sunset (Iftar) during the month of Ramadan which lasts for 29 to 30 days while other religion allows fasting on certain chosen days. In this study, only impact of Ramadan is focused. Ramadan is like ADF because both contain fast and feast periods with exception of restricted fluid intake during feast of Ramadan (Trepanowski & Bloomer, 2010). During Ramadan sleeping pattern is altered and observers are engaged in a consistent physical activity (Taravekh) after Iftar (Gilani, Davies,

& Khunti, 2014). Impacts of ADF on animal models have been well studied showing longevity (Rizza, *et al.*, 2014), Prevention and reduction of certain diseases like CVD, cancers and metabolic diseases like diabetes (Anson *et al.*, 2003; Longo & Mattson, 2014). Similarly ADF is helpful in cardiovascular health, low heart rate, blood pressure, increased heart rate patchiness and reducing post infract chronic heart failure (Anson *et al.*, 2003; Raffaghello *et al.*, 2008; Zhang *et al.*, 2005).

Currently religious fasting and its effect on human health with respect to certain metabolic and immunological diseases is a topic of interest from many years. Many patients have been checked by doctors during fasting for weight management, disease treatment and prevention. Effect of fasting on cellular level is also well established, it promote stress resistance, lipolysis and autophagy and may act as a powerful drug used for reducing of seizures, seizure associated brain harm and management of rheumatoid arthritis (Bruce- Keller *et al.*, 1999 ; Müller *et al.*, 2001). Studies indicate that religious fasting is an effective procedure of reducing obesity (Khaled & Belbraouet, 2009), weight loss, improving cardiovascular health status overall (Fontana, Partridge, & Longo, 2010; Masoro, 2005). Importantly, increase HDL, reduce LDL,VLDL and triglyceride level even in patients with type 2 diabetes are also observed in certain studies (Tiboura *et al.*, 2015). Religious fasting is helpful to reduces brain natriuretic peptide level to keep blood pressure normal and increase renal functions (Aktürk *et al.*, 2013; Trabelsi *et al.*, 2012). Proper functionality of liver is associated with fasting (Mohammed, 2011).

1.1 Aim

The present study aims to check the impact of energy Restriction on Human female physiology and gut microbiota.

1.2 Objectives

1. To evaluate the impact of Energy Restriction (Ramadan Fasting) on human female physiology.
2. To determine the variation of gut microbiota due to periodic energy restriction.
3. To analyse probiotic potential of human faecal isolates.

Chapter 2

Literature Review

2 Literature Review

The terms "**microbiota**" and "**microbiome**" have been complicated with confusion in many studies. Although many studies have depicted their distant meaning, on the other hand these terms have been used interchangeably. Microbiome is sometimes used both in context of suffix "Biome" and "Omics" (Eisen, 2015; Ursell *et al.*, 2012). However, in 2001 the term microbiota was summed up by Lederberg and McCray as *"the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space"* whereas "**microbiome**" to describe the collective genome of indigenous microbes (microflora) in a body (Lederberg & McCray, 2001; Marchesi & Ravel, 2015). In a nutshell, in this study "microbiome" refers to collection of genomes while "microbiota" refers to collection of organisms in a gut (Clemente *et al.*, 2012).

2.1 Discovery of the Human Microbiome

The first scientific evidence of microorganisms inhabiting human gut emerged in the mid-1880s, when a scientist Theodor Escherich observed a bacterium (later named E. coli) in the intestinal flora of healthy and diseased (diarrhea) children. Following this study, scientist described many other residents in human body followed by another bacterial specie *Veillonellaparvula* in 1898. Similarly, in 1900 members of *bifidobacteria* were observed. Throughout the 20th century a number of other microorganisms were isolated from different parts of body e.g. the nasal and oral cavities, skin, gastrointestinal tract etc. and characterized to be human microbiota and thus the intensive studies were carried out during the start of 21st century (Kara, 2016).

To date approximately 1000-1150 microorganisms colonize in human intestinal tract of an individual, whereas amid those, only 160 are core species. Together with bacteria, archaea, eukaryotes and viruses are also potential gut residents (Clemente *et al.*, 2012; Qin, *et al.*, 2010). In 2009, Turnbaugh et al. established that a "core microbiome" is shared amongst the individuals by comparing the diversity and composition in monozygotic or dizygotic twins and their mothers (Turnbaugh *et al.*, 2009). These microbes generally do not pose any threat are to humans, in fact they are essential for maintaining health and perform other important functions. An extensive number of studies have proven that changes in the composition of human microbiomes are correlated with numerous disease conditions, raising the possibility that manipulation of these communities could be used to treat disease (Human Microbiome project , 2016).

2.2 Human Gut Microbiota (Bacteria)

It is a topic of great curiosity & interest that everyone in keen to know what a biological individual is Metagenomics have revealed that >10% of the cells comprising our body are Homo sapiens cells while the remaining 90% are bacterial cell (Gill *et al.*, 2006 ; Hutter et al., 2015; Quigley, 2013). After culturing *E.coli* successfully, over 605 intestinal microbial species have been isolated which are also supported by phylogenetic analysis. To date, approximately 500 to 1000 bacterial species are present in gut. There are more than 50 phyla. The human gut is mostly dominated by Bacteroidetes and Firmicutes while these phyla Actinobacteria (91 species), Bacteroidetes (80 species), Firmicutes (284 species), and Proteobacteria (133 species) are present in small number (Qin *et al.*, 2010; Tremaroli & Bäckhed, 2012; Wacklin *et al.*, 2014). At genera level, the prevailing intestinal genera are: Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Ruminococcus, Peptococcus and Peptostreptococcus (Guarner & Malagelada, 2003). Whereas there are also other species like *Verrucomicrobia,* Syn*ergistetes, Planctomycetes, Tenericutes* and the *Deinococcus-Thermus* group (Rajilić-Stojanović & de Vos, 2014). These representative species are mostly considered as operational taxonomic units (OTU's). OUT's are distinctive sequence types that characterize a species but do not necessarily have a definite name, since there is no culture representative or the taxonomy of these species in not established as yet (Rajilić-Stojanović & de Vos, 2014). However, these species only account for less than 10% of the whole assembly of intestinal residents while the remaining are non-culturable.

The MetaHIT consortium reported that human gut microflora is mainly composed of three enterotypes first characterized by Manimozhiyan et al. These enterotypes are *Prevotella, Bacteroides*, and *Ruminococcus* (Wu *et al.*, 2011). The figure below shows relative abundance of different species in human gut.

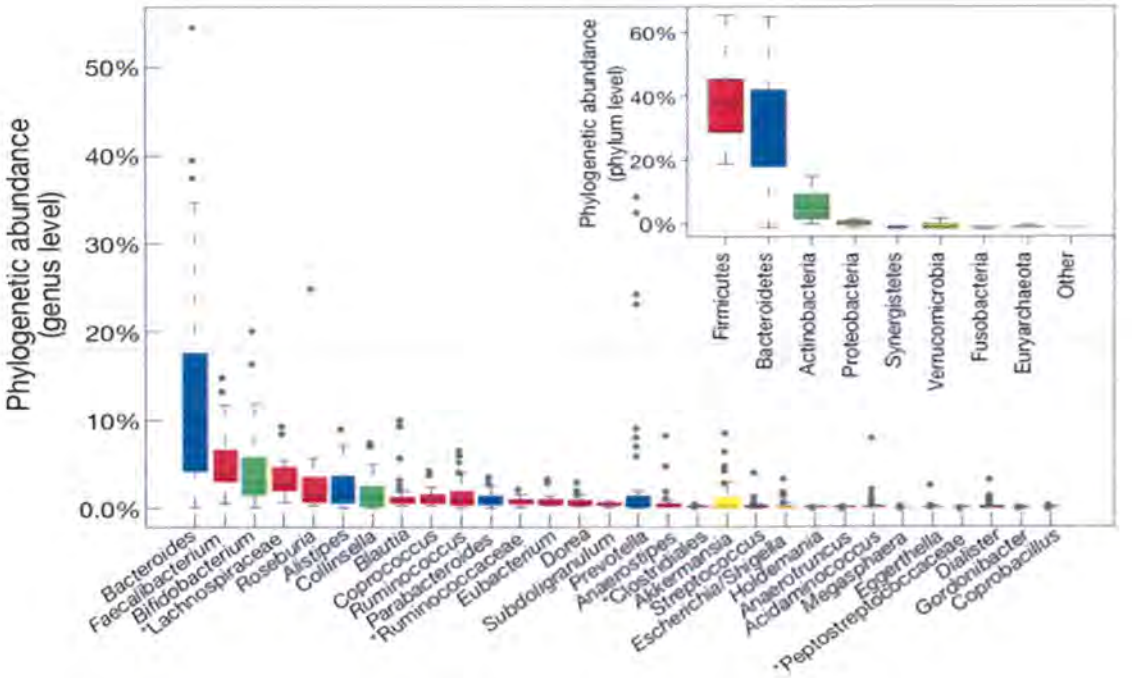


Figure 2.1: Diversity of Bacterial Species at Genera Level

(Figure taken from Armaghuman *et al.*, 2011)

2.3 Non-Bacterial Members of the Gut

2.3.1 Viruses

Although the viruses in the gut are poorly studied and characterized, the first study of the human gut virome described 1200 viral genotypes, most of which belonged to *siphophages* and *prophages* within bacterial genomes while the faecal dominating families are *Siphoviridae* and *Podoviridae* (Breitbart *et al.*, 2003). The viruses perform a protective role in maintaining the ecology and also health balance in human gut (Kernbauer, Ding, & Cadwell, 2014). Bacteriophages also play role in inhibiting pathogenic bacteria (Barr *et al.*, 2013).

2.3.2 Archaea

Archaea are present in the majority of human gut samples. The majority occurring community is methanogens (methane producers). Despite their abundance, they have low diversity. To date, only three methanogens have been isolated from human faeces: *Methano brevibactersmithii, Methanosphaera stadtmanae,* and *Methanomassiliicoccus luminyensis.* Their role in gut is to augment the efficiency of polysaccharide fermentation by preventing build-up of H_2 and other reaction end product (Dridi, 2012).

2.3.3 Fungi

The dominant members of the eukaryotic component of the human microbiome are the fungi like *Candida Tropicalis* detected as abundant pathogen. They have high diversity in human gut. An analysis of the mouse gut mycobiome has revealed 100 annotated fungal species together with 100 non-annotated species (Moyes & Naglik, 2012).

2.4 Composition of Human Gut Microbiota

The widely held gut microbiota constitutes strict anaerobes mainly facultative anaerobes. The microbial species are host specific, developing throughout a person life and susceptible to both exogenous dietary habits and endogenous modifications (Sekirov *et al.*, 2010). A study performed by Frank et al, reported that collective gut microbiota constitutes above 35,000 bacterial species (Frank *et al.*, 2007).

2.5 Stomach and Small Intestine

The stomach and small intestine has acidic and enzymatic environment accompanied by peristalsis and other gastric secretions which does not allow the growth of many bacteria. Most bacteria in stomach are either inhabitants or ingested ones. Most dominating phyla include Proteobacteria (made up of mainly, *Actinobacillus*, and *Neisseria),* Firmicutes (dominated by *Streptococcus* and *Bacillus), Helicobacter, Haemophilus* Bacteroidetes (mainly *Prevotella)* and Actinobacteria (Delgado *et al.*, 2013). Interestingly, commensal microbiota with probiotic properties have been isolated from the stomach (Ryan *et al.*, 2008).

Although the most microbial and host interaction occurs in small intestines but this area is less studied due to difficulty in obtaining samples. Presently, ileum and jejunum communities in small intestines have been characterized. The small intestine is mainly dominated by these phyla: Proteobacteria, Firmicutes and Bacteroidetes. The most occurring genera Bacteroides, Streptococcus in jejunum and Clostridium clusters in ileum (Wang, Ahrné, Jeppsson, & Molin, 2005).

2.6 Large Intestine (Colon and Rectum)

Large intestine is a microbial organ that accommodates a vast and diverse biomass of bacterial communities. It has mucosal and luminal adhered microbiota. Overall, microbiota is dominated by these phyla: Firmicutes (clusters IX, XIV and XVI), Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia e.g. Akkermansia (Walker *et al.*, 2011). No quantitative

or qualitative differences were observed between the microbial communities at different segments of the colon i-e ascending, descending, sigmoid colon, transverse and rectum segments in patients that had their bowels cleaned before the biopsies (Walter & Ley, 2011). The main functions of some important phyla are discussed in a table 2.1.

Table 2.1: Four dominant gut bacterial phyla and known metabolic functions (Ottman, Smidt, De Vos, & Belzer, 2012)

Species	Functions
Firmicutes (G+ive)	Activate metabolism of carbohydrates
Bacteriodetes (G-ive)	Helps in metabolism, digestion of complex carbohydrates, energy production etc.
Actinobacteria (G+ive)	Activate sugar and carbohydrate metabolism
Proteobacteria (G-ive)	Mostly are opportunistic pathogen

2.7 Functions of Gut Microbiota

Gut microbiota characterization is first step for elucidating its role in human health, drug responses and diseases. As each individual has its own unique gut flora therefore researches are now trying to identify certain stable patterns of microbial population in humans (Shreiner, Kao, & Young, 2015). Under healthy conditions the gut flora occurs in state of "normobiosis". The gut microflora performs many physiological functions that are discussed below (Sommer & Bäckhed, 2013). Recent studies show that human decision making activities, behaviour, memory and learning are also affected by the gut microflora (Blottière, De Vos, Ehrlich, & Doré, 2013).

2.8 Metabolic Functions

The human microbiota performs diverse metabolic functions that are necessary for proper functioning of host such as;

1. Ferments large and indigestible polysaccharide such as resistant starches from the diet with the help of enzymes resulting in energy salvage and substrates absorption for the host (Bull & Plummer, 2014).
2. The fermentation products (short-chain fatty acids) provide energy to host and promote the growth of intestinal epithelial cells (Sartor, 2008).
3. In general, SCFAs acidy the lumen thus suppressing the growth of pathogens but they also prevent colon cancer development and modulate immune responses (Short chain fatty acids (SCFAs) have nutritional, regulatory, and immunomodulatory properties (Sun & O'Riordan, 2013).
4. Gut microflora synthesize various vitamins especially vit K and vit E e.g., Folate, riboflavin, thiamine, biotin, pantothenic acid. Species of Bifidobacterium, Bacteroidetes and Enterococcus synthesize vitamins. *Lactobacillus acidophilus,* also found in yogurt and cheeses, synthesize vitamin B12, pyridoxine and niacin (LeBlanc *et al.*, 2013).

2.9 Trophic

They exert tropic effect by providing nutrients (SCFs) to the epithelium of gut, thus maintaining cell differentiation and proliferation (Sansonetti & Di Santo, 2007).

2.10 Protective

Protection against pathogen colonization and intrusion by competing for nutrients, lowering intestinal pH and producing certain substances e.g. bacteriocin, peroxides and SCFs etc. (Clemente *et al.*, 2012; Gareau, Sherman, & Walker, 2010; Guarner & Malagelada, 2003). The capabilities of the gut microbes to control infections in the host can be divided into two mechanisms; indirect inhibition through modulation of host immune systems and direct inhibition by commensal-pathogen interaction. Indirect inhibition includes colonization resistance, Nutrition competition, Immune system development, Inhibition of adhesion and direct inhibition is via production of antimicrobial molecules and by inhibiting quorum sensing (Yoon, Lee, & Yoon, 2014).

2.11 Immunomodulation

Complex and bidirectional interaction exists among gut microbiota and body's defense system. The gut microflora also controls different aspects of innate and acquired immunity, protecting the host from pathogen attacks and chronic inflammation. On the contrary, disturbances in

composition of gut microflora are linked with susceptibility to infections, immunological disorders, insulin resistance and weight gain (Diamant, Blaak, & De Vos, 2011).

a. Stimulates the production of immunoglobulin.
b. Promotes the activity of anti-inflammatory cytokines and down regulates the activity of pro-inflammatory cytokines.
c. Induction of regulatory T cell (Takagi *et al.*, 2016).

2.12 Gut-Brain Communication

The CNS and the gut are closely associated by a complex communication network. Signals from the gut via vagus nerve, modify brain functions, immune responses, mood and behaviour. Similarly signals from brain govern the functions of GI tract. Several studies showed that changes in the gut flora have an impact on the CNS (Grenham, Clarke, Cryan, & Dinan, 2011). Microbes communicate with gut-brain axis by certain mechanisms such as:

a. By releasing SCFAs which indirectly regulates production of hormones.
b. By enhancing the expression of (GABA) receptors. GABA is produced in brain from glutamate and acts as an inhibitory neurotransmitter. Binding of GABA with its receptor inhibits nervous activity. This mechanism has an application in reducing stress, pain sensitivity and anxiety level.
c. Through engagement with toll like receptors (TLRs), microbes alter the expression of chemokines and cytokines. This causes change in function of immune cells. Specific products of the immune cells interact with neurons via membrane receptors, thus modulating the nerve signaling (Grenham *et al.*, 2011).

2.13 Colonization and Establishment of Human Gut Microbiota

The microbes in our gut are there maybe by coincidence and have founded a niche to grow. They need to grow, reproduce, colonize our gut, to grow on the nutrients provided by host and to reproduce in gut (Heaton *et al.*, 1992). The gut microbiota colonizes virtually all surface of the body exposed to outside environment. Microorganisms flourish on skin and in the genitourinary, gastrointestinal, and respiratory tracts but due to availability of some potential substrates in the GIT that can be consumed as nutrient source by microbes thus, making it a preferred surface for colonization. Each organism occupies specific niche, based on availability of best substrates these bacteria utilize and thus perform a specific and different function. Crow

in 2011, mentioned that Bacteroidetes and Firmicutes colonize intestines (anerobic) because of the function they perform (fermentation) (Crow, 2011).

If no nutrient from outside would enter the GIT, the only substrate bacteria would utilize is - host excretions (saliva, mucus, gastric, pancreatic juices and bile etc.). The secretions create harsh conditions such as low pH, proteolytic enzyme and secrete anti-microbial substances. Mucins secreted from saliva and mucus from intestinal epithelium, are most dominant substrates that bacteria would utilize (Arumugam *et al.*, 2011). This section will discuss the initial to final colonization.

In 1900, Tissier studies gave the idea that in utero embryo is sterile and microbial colonization starts during the birth. The microbes come during the birth process and from the surrounding environment and the process of colonization continues until a staple and complex communities are established (Harmsen *et al.*, 2002; Palmer *et al.*, 2007). Even after a period of 100 years, it was believed that the presence of any microbe in utero is a risk for the fetus. Later on, few studies have examined the bacterial presence in amniotic fluid (Bearfield *et al.*, 2002; Rodríguez *et al.*, 2008), umbilical cord (Jiménez *et al.*, 2008), placenta (Aagaard *et al.*, 2014) and fetal membranes during healthy pregnancy (Matamoros *et al.*, 2013; Rautava *et al.*, 2012; Steel *et al.*, 2005).

In humans the gut microbiota is established after one to two years of birth, and by that time the intestinal epithelium and the intestinal mucosal barrier co-developed in a way that is tolerant to, and even supportive of the gut microorganism and that also provides a barrier to pathogenic microbes (Faderl *et al.*, 2015). The process of intestine microbiota establishment can be divided into the following steps (Clemente *et al.*, 2012).

2.14 Pregnancy and Prenatal Microbial Contact

Meconium harbors complex microbial community (Gosalbes *et al.*, 2013). A research was conducted by Collado et al, where it was reported that bacterial load increased over the time of the gestation (Collado *et al.*, 2008). Similarly, many other studies have been conducted due to recent advances in cultural dependent and independent techniques. The scientist conducted a study and observed the changes in the gut microflora that occur from the 1st (T1) to the 3rd (T3) pregnancy trimester and have assessed the potential abilities of T1 and T3 microflora to induce metabolic changes by means of germ-free mouse transfers. They provided evidences that the gut community's composition and structure are profoundly changed over the period of

pregnancy. Moreover, the third trimester microbiota induces metabolic changes in germ-free recipient mice that are similar to aspects of metabolic syndrome. These changes are associated with metabolic disease in non – pregnant women and men but may be helpful in the context of a normal pregnancy (Koren *et al.*, 2012). Generally, the diversity of gut microbiota decreases between the 1st and 3rd trimesters, but the abundance of certain types, such as the Proteobacteria and Actinobacteria, increased after birth, the children's microbiota resembled those of the mothers' first trimester samples (Koren *et al.*, 2012).

In 2013, a study characterized microbiota of meconium & faecal samples of premature infants (during first 3 weeks of life) by means of a culture dependent and independent methods. Similar results were revealed from both techniques. In meconium, phylum Firmicutes was dominant while in faecal samples phylum Proteobacteria was abundant. Culture dependent techniques showed that in meconium, Staphylococci predominates, while in feces, Enterococci and some Gram negatives (*E. coli*, *S. marcescens*, *K. pneumonia*) are abundant. Additionally, 16S r DNA based microarrays revealed the presence of *L. plantarum* and *S. mitis* in meconium and presence of *Enterococcus*, *K. pneumonia* and *E. coli* in infant faeces. Through an ascending route, vaginal microbes (linked with premature birth) can get access to the uterine environment. Though this mechanism is not yet completely understood (Moles *et al.*, 2013).

One study was conducted to check whether the maternal gut microbiota can be transferred to the fetus or not. *E. faecium* was genetically labelled and was given to the pregnant mice orally. Interestingly, genetically labelled *E. faecium* was cultured from the amniotic fluid and meconium of pups from inoculated mice as compared to the control. Hence, this study laid foundational evidence for the transmission of maternal microbes in mammals. Remarkably, in rodents during pregnancy and lactation bacterial translocation is greatly increased (Perez *et al.*, 2007).

Worldwide, many recent findings have challenged the dogma that fetus resides in sterile environment and here the question has arisen regarding the impact of microbes colonizing fetus on the pregnancy and neonate's health status. Some placental microbes like *Gardnerella* and *Prevotella* may induce inflammatory responses in new-born whereas *Lactobacillus* sp. suppress such responses (Fichorova *et al.*, 2011; Stout *et al.*, 2013). At present, little knowledge is available about the microbes that can cross the placenta and their effect on the health.

Nevertheless, high-throughput sequencing tools will be helpful in characterizing "fetal microbiome" in utero.

Nevertheless, there are conflicting findings in literature about the composition of the neonatal GIT microbiota and the factors that influence it. Some studies have showed that *Bifidobacterium* mostly dominates the gut microflora of breast-fed babies by several weeks of age (Favier, Vaughan, De Vos, & Akkermans, 2002).

2.15 Microbial Colonization

The colonization starts with a diverse microbiota both from mother and environment. Studies on neonates have shown that facultative anaerobes colonize first. Though it has less diversity but the quickly dominating species are *Bacteroides, Prevotella, lactobacilli, streptococci, staphylococci,* and *Enterobacteriaceae* (Dominguez-Bello *et al.*, 2010; Isolauri, 2012; Penders *et al.*, 2006). Upon starting nutrition, the microbiota changes depending on the type of nutrition. Different microbes will colonize in breastfeeding and formula feeding (Harmsen *et al.*, 2000). Breast milk bacteria are bifidogenic. This is not primarily lactose but also specific oligosaccharides for which *bifidobacteria* have specific enzymes resulting in dominance of *bifidobacteria* within one week of birth (Sela *et al.*, 2008; Zivkovic, German, Lebrilla, & Mills, 2011). The diversity of the bacteria increases as the child gets older. It is not clear what is driving this but gastrointestinal infections and antibiotic use will play a role in this. The species of *bifidobacteria* that colonize the gut may vary across the individual (Bergström *et al.*, 2014). Upon commencement of solid food and fruits etc. the complexity and diversity of gut microflora increases. Bacteria such as Prevotella, Bacteroides , ruminococci and *Clostridium* clusters colonize with the commencement of food (Favier *et al.*, 2002). Along with these bacteria Firmicutes and different enterobacteria species are also introduced. Diverse microbiota starts appearing at the age of 1 year to 3 years and stabilization occurs at age of 3 (Yatsunenko *et al.*, 2012). This stabilization leads to a phase where the microbiota does slowly develop further. It has been proposed that the gut flora remains stable up to 7 years of life (Biagi *et al.*, 2010). By the end of the first year of life, the microbial profile is distinct for each infant; by the age of 2.5 years, the microbiota fully resembles the microbiota of an adult in terms of composition. The microbiota of young children and adolescents is still different from that of adults and the microbiota continues to develop and change until old age, where there are again significant differences with the microbiota of e.g. 30 year old adults (Claesson *et al.*, 2011). As we grow old, the number of Firmicutes, *Bifidobacteria* and Faecal bacterium *prausnitzii*

decreases while there is an increase in the amount of *E. coli* and *Proteobacteria* (Claesson *et al.*, 2011; Mariat *et al.*, 2009). The older adult microbial profile can be distinguished from the younger adults on the basis of decrease vitamin B12 biosynthesis and microbial reductase activity along with an increase potential for stress response, DNA damage and immune compromise. Such findings propose that gut flora of older adults represent a pro-inflammatory phenotype (Lan, Kriete, & Rosen, 2013).

A study of infants from three geographical areas, United States, rural Malawi and Venezuela showed how bacterial diversity increases with age and stabilizes after about 3 years of life (Yatsunenko *et al.*, 2012).

During the first weeks of life, there is a reduced activity of TLRs, potentially allowing the necessary formation of a stable bacterial community in the gut. As the infant grows, and with the introduction of solid foods, the microbiota diversity increases, and the community converges toward an adult-like state. At the same time, the immune system "learns" to differentiate between commensal and pathogenic bacteria. By adulthood, a relatively stable community composition (but varying between different individuals) is achieved, dominated mostly by Bacteroidetes and Firmicutes. Different diseases are characterized by significant changes in the microbiota and associated changes in the production of cytokines.

2.16 Factors Affecting Colonization of Gut

Various factors affect early development of the gut microbiota. Breastfeeding vs formula feeding and caesarean section vs natural delivery are important determinants of the gut microbial development which have a long term impacts on gut microflora (Adlerberth & Wold, 2009; Lozupone *et al.*, 2013; van Nimwegen *et al.*, 2011).

Other known factors to influence colonization are: gestational age, mode of delivery i.e. vaginal birth vs assisted delivery, diet - breast milk vs formula, level of sanitation, and exposure to antibiotics (Collado *et al.*, 2012). Other less obvious, but important factors include maternal factors expressed in breast milk, host factors like genetics and innate immunity (Álvarez-Martín *et al.*, 2007; Zivkovic *et al.*, 2011).

2.17 Mode of Delivery

Soon after birth, postnatal microbial exposure occurs. Babies delivered vaginally harbours mothers' vaginal microbial communities in their gut such as species of *Bifidobacterium* and

Lactobacillus. Whereas, babies born through caesarean section acquire species of *Propionibacterium* and *Staphylococcus* from skin (Dominguez-Bello *et al.*, 2010). The microbial composition of infants born via C-section remain disturbed for several months and/or years (Abrahamsson *et al.*, 2014; Salminen *et al.*, 2004).

During vaginal delivery, vaginal microbes are transferred from mother to the infant. The microbial diversity of vagina is low, mainly consisting of Lactobacilli sp. (Aagaard *et al.*, 2012). Thus, during early days of life infant's gut is colonized by Lactobacilli, reflecting vaginal microbiota (Avershina *et al.*, 2014). During delivery, human anatomy also supports the exposure of maternal fecal microbes (Lozupone *et al.*, 2012). Members of mother are transferred through feces (de Muinck *et al.*, 2011). Studies show that the transmission of *Bacteroides fragilis* also occurs during birth (Rudi, Storrø, Øien, & Johnsen, 2012). Some recent reports show that the microbial diversity of C-section infants is low as compared to the vaginal delivery. Caesarean births have greater risk of IBD, type 1 Diabetes, celiac disease, asthma and allergies (Bager, Simonsen, Nielsen, & Frisch, 2012; Negele *et al.*, 2004).

2.18 Feeding Mode

Bacteria in the mother's milk have a significant role in the infant's gut microbial development (Fernández *et al.*, 2013). More than 700 bacterial species have been reported in mother's milk (Cabrera-Rubio *et al.*, 2012). Majorly species of Staphylococci and Streptococci are dominant in mother's milk and so are the earliest colonizers of infant's gut (Cabrera-Rubio *et al.*, 2012). Breast milk also contain complex oligosaccharides (prebiotics), favouring the growth of specific bacteria like Bifidobacterium sp. (Zivkovic *et al.*, 2011) and antibodies, strengthening infant immunity. In contrast, bottle fed infants harbour more *Clostridia* species and have increased incidence of allergy and autoimmune diseases (Iyengar & Walker, 2012). With the intake of solid food, gut microbial diversity increases. More butyrate producers will colonize the gut such as Bacteroides and some Clostridium spp. (Fallani *et al.*, 2010).

2.19 Antibiotics Intake

Previously, the use of antibiotics was generally taken in sense of disease curing. Recently many studies perform on human gut regarding antibiotics usage have implicated their role both long and short term in sustaining gut ecology. The chief role of gut microbiota is prevention of pathogens colonization. It was illustrated approximately four decades ago that antibiotics

intake might result in competitive exclusion machinery that caused *Salmonella* infection soon after antibiotic therapy (Lakdawala *et al.*, 2013).

Recent studies have been conducted using high-throughput sequencing technologies to characterize the long-term effects of antibiotics (Panda *et al.*, 2014). However, many other studies on the long-term effects of antibiotic usage have shown that microbiota does not show complete recovery three months after stopping treatment.

Most important alterations in the gut microflora in reaction to antibiotics are: diminished taxonomic diversity and persistence of alterations in a considerable fraction of individuals. It has been reported that the effects of even short-term use (7days) of broad spectrum antibiotics with predominant anaerobic coverage e.g. Clindamycin could remain for two years, with a persistent non-recovery of the diversity of Bacteroides. In the same way, a short course of *H. pylori* eradication with clarithromycin comprising triple therapy caused a dramatic decrease in the diversity with thousand folds increase in the ermB resistance gene. It persisted for over four years in a proportion of these patients, while it recovered in the others. The consequence of ciprofloxacin, which has predominantly Gram-positive coverage, is relatively short-lived with abrupt decrease of *Ruminococcus* spp. Another current study evaluated the role of short course (7 days) of ciprofloxacin and beta-lactams. The study indicated the decline of microbial diversity by 25% and the core taxa from 29 to 12% with an increase in the *Bacteroidetes-Firmicutes* ratio. The main concern that stems out of use of broad-spectrum antibiotics, besides changes of the normal microbial diversity, is the phenomenon of propagating the resistance strain via horizontal gene transfer (HGT). Bacterial species are proficient of transferring mutant genetic information across different species via conjugation, phage transduction and natural transformation. The gene transfer could also be through transposons and integrin. Interestingly, it has been shown that among different environments, the human gut associated microflora has 25 times more possibility of having HGT. This would result in development of a reservoir state of resistance genes, and consequently mandates extreme care in the use of broad spectrum antibiotics.

The acute effects of antibiotic treatment on the native gut microbiota range from self-limiting "functional" diarrhea to life-threatening pseudomembranous colitis. The long-term consequences of such perturbations for the human–microbial symbiosis are more difficult to

discern, but chronic conditions such as asthma and atopic disease have been associated with childhood antibiotic use and an altered intestinal microbiota (Panda *et al.*, 2014).

In mice models, antibiotic treatment altered the gut microflora and induces some metabolic changes like increased production of fatty acids. Studies show that in animal feed even low antibiotics doses can cause modification in copies of carbohydrate metabolism genes. Recent studies show that antibiotics can induce gut dysbiosis, which aggravates allergic inflammation by stimulating M2 macrophages polarization. This polarization increases the Candida species in cecum, which is due to the decrease in *Lactobacillus* species (having antifungal properties). The results of these studies suggest that probiotics and prebiotics can be used to treat allergies by enhancing *Lactobacillus* species within the gut (Willing, Russell, & Finlay, 2011).

2.20 Diet

Diet has a major influence on shaping the structure of the microbiome. Changes in diet influence the relative abundance and composition of the microbiota almost immediately, but for these compositional changes to become stable, the diet has to be long term (Turnbaugh, Ridaura *et al.*, 2009; Wu *et al.*, 2011)

Generally, diet intake rich in fruits, fibres and vegetables is linked with a high microbiota diversity. People taking such diet have abundant insoluble carbohydrate metabolizing organisms from Firmicutes phylum such as *Ruminococcus bromii, Roseburia Eubacterium rectale* (David *et al.*, 2014; Walker, *et al.*, 2011).

In humans, an increase in the ratio of Bacteroidetes-Firmicutes is observed when restricting the calorie/carbohydrate content. A high fibre diet is linked with an increase in Bifidobacterium, Lactobacillus-Enterococcus, and Ruminococcus was also shown to increase in abundance in subjects on a resistant starch diet. High fat diet can profoundly alter the composition of the microbiota towards an enrichment of Firmicutes (Faith *et al.*, 2011) and high levels of casein have been shown to decrease the abundances of specific taxa (e.g., *Eubacterium rectale, Marvinbryantia formatexigens,* and *Desulfovibrio piger)* (Shen, Zhao, & Tuohy, 2012).

Studies have depicted seasonal and geographic differences in gut microbiome. However, these variances were also linked with changing diet pattern. For example, rural African children had abundant Prevotella, while European children had higher proportions of Bacteroides. Even though Prevotella and Bacteroides are taxonomically and functionally alike, but high

abundance of Prevotella shows that agrarian diet that was taken by the African children. On the contrary, the children from Europe consumed a western diet rich in animal protein, sugar, starch and poor in fibres, which is marked by the higher abundance of Bacteriodes (De Filippo *et al.*, 2010).

Individual members of the microbiota and their consortia have been shown to be dependent on the nutrient composition of the diet. Prevotella grows better on carbohydrates; dietary fiber provides a competitive advantage to Bifidobacteria and Bacteroidetes has a substrate preference for certain fats. Some specialist microbes, e.g. mucin degrading bacteria such as *Akkermansia mucinophila*, thrive on secreted carbohydrates provided by host cells. Other butyrate producing microbes, e.g. Roseburia spp., fare better when they are delivered polysaccharide growth substrates in the diet (Wu *et al.*, 2011).

Microbes can induce cravings, can alter eating behavior, mood and behavior. Although certain Lactobacillus appear to reduce anxiety, colonization of the gut with the pathogen *Campylobacter jejuni* increased anxiety-like behavior in mice.

Bacteria face numerous challenges when colonizing the mammalian GI tract, and for the development of a beneficial microbiota (undertaking commensal, mutualistic, or symbiotic relationships with the human host), these must be overcome without reducing host fitness (Ley, Peterson, & Gordon, 2006).

2.21 Gut Dysbiosis

Despite performing many substantial and dominant functions, the gut bacteria may post threat to host and can cause many diseases. At the beginning of 21st century, after the bowel toxaemia theory, Elie Metchnikoff suggested the production of certain noxious substance by gut microbiota having host detrimental effects (Metchnikoff, 2004). These suggestions lead the concept of gut dysbiosis which is defined as a condition of microbial imbalance in each habitat of a body. Researchers have discovered that microbial imbalance causes compromised gut barrier, leading to certain diseases such as IBD, CVD, NAFLD and other metabolic syndromes. Metabolic diseases include obesity, diabetes mellitus type 2, and atherosclerosis etc. (Kau *et al.*, 2011). In this section, only certain metabolic syndromes are discussed.

Studies done on gut microbiota of normal and diseased (metabolic) people have shown a remarkable difference in gut microbiome. Certain inclusion criteria are implied to consider a

metabolic syndrome. These criteria include co-occurrence of any three of the following five conditions (abdominal obesity, low levels of HDL cholesterol, elevated blood pressure, high levels of triglyceride, high blood sugar after fasting). Individual suffering from metabolic syndromes harvest more energy harvested from diet and a decrease in beneficial bacteria thus causing an overall less diversity and reduction of some important residential bacteria leading to dysbiosis (Kau *et al.*, 2011; Okada *et al.*, 2013).

2.22 Gut and Obesity

World report shows that approximately 500 million adults are over-weight having BMI of 25.0-29.9kg/m^{2}and 250 million are obese having BMI $\geq$ 30kg/m^2) (Bouchard, 2000). This lead the development of disorders related to obesity. These disorders have been found related to role of gut microbiota. In year 2005, it was initially stated that obese individuals have different gut microbiota compared to obese ones. It was investigated and found that Firmicutes are higher than Bacteriodes in obese mice in comparison to lean mice (Wolf, 2006). A study conducted by Turnbaugh et al, proved that transplantation of a gut microbiota from chronically obese mice (cecal) into adult GF mice (Turnbaugh, Bäckhed, Fulton, & Gordon, 2008). The recipient from obese became obese and the lean one became lean despite feeding them with same diet and keeping in same environment. Gut microbes influence obesity through increased dietary energy harvest, effects on the feeling of satiety after eating, increased fat storage, and systemic inflammation (De Angelis *et al.*, 2014).

The possible mechanisms for the overweight regulating effect of microbiota arise as follows: the gut microbiota enables host to enhance the hepatic production. This enables hydrolysis of indigestible polysaccharides to easily absorbable monosaccharides, and activation of lipoprotein lipase with consequent excessive storage of liver-derived triglycerides in adipocytes (Bäckhed *et al.*, 2004). These processes boost weight gain, which may be counteracted by modification of the gut microbiota by probiotics with a potential to balance the low-grade inflammation associated with obesity (Hamad *et al.*, 2009).

Diet also plays an important role in regulation of gut microbiota and obesity. Recently, found that when a mouse was given fat rich diet, then number of Firmicutes was increased and this modification was rapidly changeable. Thus, diet alters certain phylotypes in gut bacteria. Besides, mice gut microflora is adaptive and has ability to respond to the changes in diet pattern. Similarly, the number of *Akkermansia muciniphila* (mucin degrader) was dramatically

decreased (about 100-1000 folds) in obese mice having obesity either genetically or diet-induced (Everard *et al.*, 2013). A negative correlation exists between the proportion of *A. muciniphila* and type 1, type 2 diabetes and body weight (Hansen *et al.*, 2014).

Several other environmental factors like an imbalance in caloric consumption and its expenses, use of drugs and sleeping pattern can also influence the genetic expression of obesity (Tsai, Cheng, & Pan, 2014).

2.23 Diabetes Mellitus Type II

Diabetes occurs when pancreas does not produce sufficient insulin or when the body does not use the insulin properly. Diabetes mellitus type 2 is the body's ineffective use of insulin. (Organization, 2014). Individuals suffering from type 2 diabetes are identifiable by their gut microbiota. Type 2 diabetics have increase number of opportunistic pathogenic bacteria, and a decrease number of beneficial bacteria (Fukuda & Ohno, 2014). Larsen *et al* found high Firmicutes while low Bacteroidetes number in the gut flora of type 2 diabetic patients. As well it was observed that an encouraging association among the plasma glucose level and Bacteroidetes to Firmicutes ratio along with Bacteroidetes-Prevotella group to *C. coccoid – E. rectale* group ratio occurs (Larsen *et al.*, 2010; Ottman *et al.*, 2012). A study done in China found diabetes associated with gut microbes (Fukuda & Ohno, 2014).Thus, gut dysbiosis correlates to type 2 diabetes. Moreover, Beta proteobacteria are more in diabetic patients than in non-diabetic controls. Such reports suggest that Proteobacteria and Bacteroidetes may stimulate type 2 diabetes by inducing inflammatory responses. Since they have high level of endotoxin LPS because of their gram-negative nature. Memberz et al, have studied the influence of depleted gut microflora on glucose tolerance in obese mice, as a model of type 2 diabetes (Membrez *et al.*, 2008). In this model, antibiotic treatment (for 2 weeks) causes reduction in the proportion of aerobic and anaerobic microbes. These antibiotic treated obese mice had less plasma LPS concentration and liver triglycerides while more plasma adiponectin concentration and liver glycogen when compared with non-treated obese mice. Thus, it signifies the role of gut microbial depletion in obese mice, for improving of glucose tolerance. As with obesity, exposure to certain antibiotics increases the risk of diabetes (Boursi, Mamtani, Haynes, & Yang, 2015).

2.24 Atherosclerosis and Cardiovascular Disease

Cardiovascular disease (CVD) accounts for about 30% of deaths worldwide. Major risk factors are diabetes and obesity. Studies done on plaques isolated from arterial walls showed the presence of bacterial DNA from the genera Chryseomonas, Veillonella, and Streptococcus (Koren *et al.*, 2011).While metabolomics studies showed the presence of trimethylamine N-oxide (TMAO), choline and betaine in plasma to be the risk factors of atherosclerosis. Mechanistic studies have revealed the microbial metabolism of phosphatidylcholine produces TMA and liver it is absorbed and converted to TMAO. Choline supplementation has resulted in more plague formation and reduction of the gut microbiota by treating with antibiotics increased symptoms (Wang *et al.*, 2011). To understand the role of these phospholipid associated molecules, murine model of atherosclerosis (apoE deficient mice) was utilized. In apoE deficient mice, positive correlation was found between the aortic lesion area and plasma TMAO level.

Furthermore, the expression level of hepatic Flavin monooxygenase (transforming trimethylamine to its oxide form) was positively correlated with TMAO levels in plasma. Treatment of apoE deficient mice with antibiotics, caused reduction in the plasma TMAO level and atheroma's size occurred. Such results suggest that in apoE deficient mice, gut microflora have a significant effect on atheroma development (Wang *et al.*, 2011). Recent studies on mice show that microbiota produces TMA and TMAO from dietary L-carnitine. This TMAO stimulate atherosclerosis (Koeth *et al.*, 2013). 16S rRNA pyrosequencing revealed that cecal microflora of mice, having diet supplemented with L-carnitine, was enriched with genus Prevotella and is positively correlated with the level of TMAO in plasma. Studies comprising the correlational analysis of TMAO level in plasma and fecal microflora composition showed that plasma TMAO level was higher in subjects with Prevotella enterotype in comparison to those subjects with Bacteroides enterotypes.

Metagenomic analysis revealed that in atherosclerosis patient genus Collinsella was abundant while genus Eubacterium and Roseburia were abundant in healthy individuals (Karlsson *et al.*, 2012). Furthermore, the faecal microbiome in symptomatic atherosclerosis patients was enriched with genes involved in peptidoglycan synthesis whereas, phytoene dehydrogenase gene was depleted. According to this, atherosclerosis patients had lower β-carotene levels. These results hypothesized that atherosclerosis patient's inflammatory status can be linked with variations in gut microflora.

2.25 Modulation of Gut Microbiota

If one accepts the reality that the microbiota plays significant functions, then it is reasonable to think of ways for regulation and preserving 'healthy' microflora configuration or to modulate them to prevent pathogenesis and to overcome diseased conditions.

There are many therapies, methods that can modulate gut microbiota. Some of which are prebiotics/probiotics intervention, diet intervention, faecal transplantation and bariatric surgery etc.

2.26 Modulation with Probiotics and Prebiotics

One way to reshuffle the gut microbiota is the use of probiotics. Commonly used probiotics are the strains of Lactobacilli and Bifidobacteria In 1907, Élie Metchnikoff firstly proposed that intake of LAB could remove putrefactive bacteria in colon and also helps in maintaining normal functions (Anukam *et al.*, 2008). A huge literature supports the use of probiotics and their effect to beneficially change intestinal microbial diversity in rodents. Similarly, the use of probiotics balanced serum insulin level in pregnancy and prevent insulin resistance in glucose intolerant or diabetic patients (Andreasen *et al.*, 2010). Researchers found that the use of probiotic yogurt (containing mainly *Lactobacillus* and *Bifidobacteriumi)* reduced total cholesterol, low density lipoprotein cholesterol, fasting glucose and glycated hemoglobin in type 2 diabetic patients (Ejtahed *et al.*, 2012). Following this, numerous studies have been conducted to show the role of probiotics. A study conducted on gnotobiotic mics showed that intake of fermented milk product (FMP) enhanced the expression of enzymes involved in numerous metabolic pathways mostly of carbohydrate metabolism (McNulty *et al.*, 2011).

Currently, a study has been shown that use of transglucosidase which form oligosaccharides (a type of probiotic) in intestinal tract promote weight loss and blood glucose level in type 2 diabetic patients (Sasaki *et al.*, 2013). Tables below show the effect of probiotic supplements in mice and humans. Tables below shows the effect of probiotic supplements in mice and humans.

Table 2.2: Probiotic supplements and their effects in humans (Fukuda & Ohno, 2014)

Supplemented Bacteria (healthy-overweight)	Changes in humans
Lactobacillus gasseri SBT2055	Decrease abdominal visceral and subcutaneous fat
L. rhamnosus GG (in infant formula – 6 months)	Enhanced growth and high weight gain; prevent excessive weight gain in kids

Table 2.3: Probiotic supplements & their effects in mice (Fukuda & Ohno, 2014)

Supplemented Bacteria	Changes in mice
Lactobacillus plantarum PL62	Reduce weight gain and concentration of glucose in obese mice
Lactobacillus paracasei F19	Reduce total body fat and decrease amount of triglycerides in mice given diet having high fat
Lactobacillus reuteri ATCC4659	Decrease weight gain, adipose, and liver weights
L. acidophilus NCDC13	Enhance *Bifidobacteria* in feces; did not decrease adiposity

Similarly,prebiotics affect the representation and metabolic profiles of probiotic species or entrenched themembers of gut microflora. Prebiotics are fibers from plants which are obtained from unprocessed or slightly processed plant foods. Inulins (polysaccharides) derived naturally from plantspromote growth of Bifidobacteria, which reduce body weight gain, improve glucose homeostasis and improve obesity related inflammation (Fukuda & Ohno, 2014). Gut bacteria that are most beneficial to humans require plant fiber for their nutrition (Velasquez-Manoff, 2015). Such finding shows that selected and tested prebiotics and probiotics can modulate gut microbiota.

2.27 Feacal Transplants

Feacal transplants is a type of probiotic therapy which is being used from 4th century to cure diarrhoea. Researchers showed upon transplanting gut microbes from an obese human into GF mice their weight was increased and vice versa (Li *et al.*, 2015).Moreover diabetic individual

receiving fecal transplants from healthy ones showed an increase sensitivity in insulin and more butyrate producing microflora. This suggests that a diabetic therapy can be developed bytransplanting fecal from healthy donor (Bates *et al.*, 2014). Thus, fecal transplant may aid in curing certain diseaes which are otherwise untreatable. Infections caused by *Clostridium difficile* were treated with 90% success by fecal transplants.

2.28 Antibiotic Treatment

Antibiotic treatment has shown some beneficial effects in host especially it helps to maintain intestinal integrity. For example in a animal experiment with administration of two antibiotics together, norofloxacin and ampicillin, showed enhanced fasting glucose level, inhibit glucose intolerance and prevent insulin sensitivity. These changes were linked with decreased blood lipopolysaccharides and liver triglycerides, with changed gut microbial structure (Membrez *et al.*, 2008). Similarly, rifaximin increased the growth of Lactobacillus. Rifaximin showed decresed mucosal inflammation induced by water avoidance stress.Antibiotic (ampicillin and neomycin) increased intestinal permeability in HF diet mice. Overall these stated studies indicate that antibiotics can modulate the gut microbiota leading to increases intestinal integrity (Cani *et al.*, 2008).

2.29 Modulation with Weigh Loss Interventions

Weight loss and metabolic improving profiles includes energy restriction, bariatric surgery, physical activities, medications and life style modification etc. (Vetter *et al.*, 2013). Present data suppose that these effects might arbitrate the intestinal flora. A research that investigated the effect of energy restriction (fat and carbohydrate) along with exercise in adults resulted in increased number of *Bacteroides fragili s*and *Lactobacillus* group while reduced number of *Bididobacterium longum* and *Bifidobacterium adolescentis* clusters. It was mentioned that weight loss upto 4kg was due to change in intestinal floral structure (Santacruz *et al.*, 2009). Nadal et al also reported the same findings that individuals who lost weight up to or more than 4 kg had decreased number of *Clostridium histolyticum* and *E. Rectle* (Nadal *et al.*, 2009).Deep metagenomics sequencing was used in a study of 49 individuals to assess the microbiota. Results showed that energy restriction of high protein diet caused higher diversity in individuals who previously had less diversity (Cotillard *et al.*, 2013). Similarly, bariatric surgery is an efficient method to r educe weight in severely obese individuals and reduce the risk of diabetes and cardiovascular disease (Sjöström *et al.*, 2004; Sjöström *et al.*, 2007).

2.30 Energy Restriction (Fasting)

Energy restriction is particularly described as a cutback in nutritional energy utilization well below the extent of energy or calories that would be taken if freely available i.e for humans' reduction from 20 to 40 % (Bales & Kraus, 2013). It can be constant generally termed as caloric restriction or intermittent (short term starvation) termed as fasting (Madeo, Pietrocola, Eisenberg, & Kroemer, 2014). Fasting as a well-being cure has been sued to be a valued healing way for long-lasting as well as severe diseases in many areas of the world (Buchinger, 1932; Fahrner, 1991). In recent time, modified fasting achieved rising fame in Germany as a self-caring way for health and cure just by changing the life style (Härtel & Volger, 2004; Lützner, Million, & Hopfenzitz, 2002). Importantly, the logics for fasting invoke both theological and therapeutic reasons (Michalsen & Li, 2013). In classical European medication, fasting was a well-known remedy since old age Greed Hippocratic school and then after considered by numerous European medical institutes for therapy of many diseases (Lützner & Wilhelmi de Toledo, 1998). The use of fasting in the background of the medical therapy pursued factual conclusions that infections and diseases have been frequently cured. European physicians such as Buchinger, Kraus, and Mayr (Buchinger Sr, 1959; Krauss & Hartmann, 1963) used fasting as a medical tool and the concluding results attracted huge amount of patients in 1950. Fasting treatments were refined and authorized in many fasting specified clinics, thus implanting well defined times of changed or subdual fasting in a complete living way scenarios. In fasting it has been seen that when energy is taken below threshold e.g. 500 kcal/day leads to activation of neuroendocrine system and promote well metabolism. In phase I stores of glycogen get mobilized, in phase II (fasting period of 24h) lipolysis starts to metabolize fats and in phase III (after fasting, starvation) proteolysis starts to metabolize proteins (Owen *et al.*, 1998). Based on energy intake level fasting is of two main types 1) Caloric restriction (CR); lowering the utilization of food, deprived of resultant in malnutrition, to a reduced amount of what an organism would eat if provide freely. Total Caloric Desistance (TCD) like intermittent fasting, alternate-day fasting (ADF), routine periodic fasting, or intermittent energy restriction (Rizza *et al.*, 2014). Numerous religious followers join phases of fasting into their customs including Muslims, who fast from sunrise (sehr) to sunset (iftar) during the holy month of Ramadan which lasts between 28 and 30 days, and Christians, Jews, Buddhists, and Hindus, who usually fast on chosen days of the week or in a schedule of the year. Ramadan is similar to ADF because both contain fast and feast periods with exception of restricted fluid intake during feast of Ramadan (Trepanowski & Bloomer, 2010).

2.31 Ramadan Fasting

Fasting is a social exercise including refraining from or constraint of solid and liquid diets, water or combinations of foods; globally adopted by numerous religion's believers. The best known example of fasting is Ramadan Fasting (Kul, Savaş, Öztürk, & Karadağ, 2014). Ramadan is an Arabic word explained in Arabic as "*sawm*", exactly sense "refraining from" (Gilani, *et al.*, 2014). Worldwide, as a spiritual responsibility every adult Muslim do daily fast regularly for 29 or 30 days in the holy month of Ramadan each lunar year. In the days of Ramadan from dawn to dusk drinks, nutrients, smoldering, sex, oral medicines, inhalations or venous dietary solutions are not allowed. Two meals are taken at nigh time. One is taken and finished before dawn called Suhour and second one is taken soon after sun set called iftar. Fasting times of each day differ according to time and terrestrial position. Moreover, because of its possible beneficial effects, including increased vigilance and mood enhancement, fasting has been advocated as a form of therapy with various specified procedures and with recently revealed mechanisms (Fawzi *et al.*, 2015).

2.32 Health Benefits of Ramadhan Fasting

There is extensive literature describing health benefits of Ramadan fasting on various aspects (Patterson *et al.*, 2015). A meta- analysis of thirty-five investigational studies was carried out to check the effect of Ramadan on body weight (Sadeghirad *et al.*, 2014). 95 % subjects were seen to reduce weight upto 1 to 1.24 kg while men were seen to lose more weight compared to women. Similarly, additional metagenomics study was conducted including 1.476 normal adults to check fats level after Ramadan (Kul *et al.*, 2014). It was seen that after Ramadan the level of total cholesterol, LDL and triglycerides was decreased significantly while hypoglycemia and ketoacidosis were observed in diabetic patients (Shaeesta, Prabhuji, & Shruthi, 2014). Adiponectin were seen to be regulated due to Ramadan fasting when 62 years old individuals were included in the study (Gnanou *et al.*, 2015). Anti-oxidative and Anti-inflammatory levels were also investigated. Fifty individuals were included in study and IL-1 B, IL – 6 and TNF – α checked after Ramadan. Significantly change was observed (Patricia *et al.*, 2013).

Chapter 3

Methodology

3 Methodology

This study was done at Microbiology Research Laboratory (MRL), Department of Microbiology, QAU and entitle as:

"Evaluation of the Impact of Energy Restriction (Ramadan fasting) on Human Female physiology and Gut Microbiome"

3.1 Population Selection

Twenty-five healthy females between age 25-35 and observing fasting from 7th June to 6th July 2016 were recruited for study. Their diet and lifestyle patterns were recorded. All individual voluntary participated in the study and prior permission was sought from all the participants. They were ensured that their data would be kept confidential.

Table 3.1: Informed Contest for Selection

- Name
- Id
- Age
- Gender
- Height
- Weight
- Body Mass Index (BMI)
- Smoking status
- Cholesterol
- vLDL cholesterol
- Serum LDL Cholesterol
- Serum HDL Cholesterol
- Gastrointestinal Disease
- Diarrhea (in last 1 month)
- Antibiotic consumption
- Supplement consumption
- Laxatives consumption
- Use of weight reducing agents
- Exercise / any other regular physical activity

3.2 Inclusion Criteria

Forty female volunteers were initially selected for the study. Selection criteria were based on health status, fasting status and other factors which are non-smokers, no antibiotics intake for

last month, no intake of laxatives for last month, no gut associated diseases, no exercise and supplements intake.

3.3 Exclusion Criteria

Exclusion criteria contains individuals not fasting, unhealthy, taking antibiotics or having IBD or any other disorder and taking any supplements consumption while fasting for the whole Ramadan month were excluded from study.

3.4 Faecal Sample Collection

Stool sample was collected from selected participants two days before Ramadan i-e 5th June & 6th June, 2016 and 5 days before Eid (1st or 2nd July). All samples were collected in sterile collection containers and then kept at -80 C temperature (Escobar, Klotz, Valdes, & Agudelo, 2014; Wu *et al.*, 2010).

3.5 Blood Collection and Analysis

10ml blood was taken twice before and at the end of Ramadan in blood collection tubes (EDTA & Gel tubes). Soon after collection, samples were processed. Serum was separated and was kept at -20°C till analysis. Different parameters were recorded from blood analysis which includes serum total cholesterol level, serum triglycerides level (TG), high density lipoproteins (HDL) levels, low density lipoproteins (LDL) levels, very low density lipoproteins (VLDL) levels.

3.6 Faecal Sample Processing

After sample collection, immediately each sample was separated into two portions. Of that one part (1Kg) was taken and serially diluted in phosphate buffer saline (PBS). The dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} were then poured and spread on different culture media for isolation of gut microbiota. The other portion was stored at -80°C for DNA extraction (Castillo *et al.*, 2006).

3.7 Culture Media Selection

Five different growth media were selected for culturing/isolation of Gut microorganism. Media were purchased from Oxiod manufacturer and prepared per instructions. For overall microbial growth or for non-selective enumeration BHIA (Brain Heart Infusion Agar was used. MRSA (de Man Rogosa Sharpe Agar) with 5.4 pH was used as a selective media to isolate *Lactobacillus spp.* M17 was used to isolate Enterococcus, Streptococcus and *Lactococcal spp.*

MacConkey agar was used to enumerate Enterobacteriaceae and OGA (Oxytetracycline Glucose Agar) was used for isolating yeast.

3.8 Culturing of Diluted Sample

A part of 0.1 ml or 100µl from each dilution was spread with sterile glass spreader on plates of culture media. Dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were spread on MacConkey and OGA media plates, dilutions 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were inoculated on MRSA and M17 media plates while dilutions 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}were plated on BHIA plates. Then spread samples were incubated at 37°C for 48 – 72hrs (Lim, Kim, & Lee, 2004).

3.9 Calculating Colony Forming Unit (CFU/g)

After incubation at specific temperatures, all plates were examined for microbial growth, color, size, and morphology of the microbial colonies were observed and recorded. Total number of colonies on each plate was recorded by using colony counter. On an average, 30 – 300 colonies were used for actual colony count. Microbial count of faecal samples was calculated by using CFU formula, colony forming unit/g of faecal material.

$$CFU/g = (number\ of\ colonies \times dilution\ factor) \div volume\ plated\ in\ ml$$

3.10 DNA Extraction for Metagenomic Studies

DNA was extracted from stored stool samples using Favor prep DNA isolation mini kit under trade name Favorgen. The kit is specifically designed for DNA extraction from stool. The protocol is followed by taking 100 mg of stool in Eppendorf tubes in which 200 mg glass beads were added. Following this, 300 µl of SDE 1 buffer and 20 µl proteinase K were added to disrupt microbial membranes. Afterwards the solution is vortexed at high rpm for 5-7 minutes and then firstly incubated at 70 °C for 10 minutes and then for 5 more minutes to lyse Gram positive cells. To ensure proper homogenization, sample was vortexed thrice during the period of incubation. After addition of 100µl SDE2 buffer, the sample was kept on ice pack for 5 minutes and then centrifuged at 14000 rpm for 5 minutes. Supernatant was transferred to another Eppendorf with addition of SDE3, 200µl buffer. The mixture is incubated, centrifuged and again transferred to Eppendorf. 1 µl RNase of concentration 100mg/ml was transferred to the supernatant for RNA degradation. After the removal of drops through spinning, 250µl SDE4 buffer and 250µl cooled ethanol were added and mixed via pulse overtaxing. Sample was transferred to collection column at centrifuged maximumly. Then the column was transferred to another collection tube, flow - through was discarded columns were transferred

to next clean collection tubes and flow-through was wasted. Impurities are washed away using 750µl wash buffer and then centrifuging then at maximum speed for 2 minutes. This step was repeated. For drying the column and avoiding residual contamination collection column was centrifuged for additional three minutes.

Finally, the column was shifted to elution tube following addition of 70µl elution buffer in its centre. To ensure complete absorption of elution buffer into the column, centrifugation was performed. DNA was eluted and stored at -20°C.

3.10.1 Gel Electrophoresis

For confirmation of quality of DNA, gel electrophoresis was carried out. 1 g of agarose gel was added to 90 ml distilled water & 10 ml TBE buffer for preparation of one percent gel. Mixture was well heated in microwave for proper mixing. Ethidium bromide was added in the gel after cooling down the get. Gel was discharged to the moulding dish having comb and was left to solidify. The dish was transferred to gel tank having 1X TBE buffer. Following the detachment of comb, 2µl bromophenol blue dye was added in 3µl sample. The sample was mixed and was laddered carefully in the wells. The sample was run on gel at 120 volts for 35 mins and 400mA. Finally, the gel was examined under UV.

3.10.2 DNA Quantification

DNA was quantified and purified by Nano Drop 1000 spectrometer.

3.10.3 Metagenomic Analysis

V4 region of 16S rRNA gene was amplified by PCR. Frequently used primer 515F (GTGCCAGCMGCCGCGGTAA) was selected and amplification was carried out in three replicates using reaction volume of 20µL (Caporaso *et al.*, 2011). Hot Star TaqPlus Master Mix (Manual & QIAquick) and sample were used for the preparation of reaction volume. PCR was carried out at these settings: early denaturing, 94°C for 3 minutes, then tailed by 30 cycles denaturation at 94°C for 30 seconds. Annealing at 53°C for 40 sec. finally amplification at 72°C for 1 minute. After the entire process, confirmation of the amplification and quality of the gene fragments were tested in 2% agarose gel. Then per molecular weight and concentration of DNA all the samples were combined with each other and were purified using calibrated Ampure XP beads. Then following the Illumina Tru Seq DNA library preparation protocol, libraries were prepared via Nextra DNA sample prep kits. Sequencing were done at MR DNA (Shallowater, TX, USA) by using Miseq sequencer. For analysis of the data, MR DNA analysis pipeline was used (MR DNA, Shallowater, TX, USA). Errors were minimized and noises were

removed. Finally, Operational taxonomic units (OTUs) were generated, and chimeras were excluded. OTUs were further refined by clustering at 3% divergence (similarity 97%). Final OTUs were taxonomically classified using BLASTn against curated database derived from Green Genes, RDPII and RDPI (DeSantis *et al.*, 2006).

3.11 Identification and Characterization of Probiotics from Selected Samples

3.11.1 Sub-culturing of Probiotics

For isolation and characterization, MRSA and M17 media were used. After incubation for 24 – 48 hours, isolated colonies were taken and were streaked on MRSA and M17 media and were again incubated for 48 hours at 37°C.

3.11.2 Preservation of Strains for Long Time

Isolated pure probiotics were stored and preserved in glycerol stock solution. Pure isolated were incubated in 700µl Trypticase soy broth (TSB) and then incubated for 24 hours. After incubation, it was shifted to 1.5 ml Eppendorf having 300µl sterile glycerol. Glycerol were sterilized by putting in hot oven at 180°C for one hour. Probiotic containing suspensions were then stored in 4°C refrigerator.

3.11.3 Microscopic Examination

The pure isolates of probiotics were examined microscopically.

3.11.4 Gram's Staining

A drop of normal sterile saline was placed on clean dried glass slide. Bacterial colony was picked and mixed with normal saline by using inoculating loop. A thin smear was made and after air fixation, it was heat fixed. Initially, smear was stained with crystal violet for one minute and washed with water. To fix the crystal violet colour, two drops of Gram's Iodine were added for 45 seconds and washed with water. Consequently, two – three drops of 95% ethanol were added for decolourization. Lastly, few drops of safranin were added for 45 sec. The stained slide was dried through air and was examined through microscope at 100X using immersion oil.

3.12 Biochemical Characterization of LAB

To characterize biochemically catalase, oxidase and lactose tests were performed for intestinal bacteria.

3.12.1 Catalase Test

Catalase test detects if the organism carries a "catalase" enzyme that has ability to detoxify the super oxides and convert H_2O_2 into H_2O and O_2. To carry out the test, a smear of 24 hours freshly cultured bacteria was made. H_2O_2, 2-3 drops, was added. The test was considered positive when bubbles were raised by adding H_2O_2 while no bubbles meant negative.

$$H_2O_2 \longrightarrow H_2O + O_2$$

Bubbles: Catalase positive (+)	**No bubbles: Catalase Negative (-)**

3.12.2 Oxidase Test

Oxidase test is performed to confirm the presence of cytochrome oxidase enzyme. Dimethyl-p-phenylenediamine-di-hydrochloride was a reagent used to perform the test. Upon oxidation, the colourless reagent presents a blue colour. Then a filter paper was soaked into oxidase reagent. A colony was picked with sterile loop and then rubbed against the reagent. Change in colour indicates a positive test and if it remains colourless that means the test is negative.

Colorless – dark purple: Positive result	**No colour change: Negative result**

3.12.3 Lactose Utilization Test

Lactose utilization test was conducted to check if the isolates are capable to utilize lactose or not- which is a property for confirmation of probiotics. Nutrient broth was prepared per manufacturer's instructions and 1% lactose was augmented to the broth. Phenol Red was added as a pH indicator before autoclaving the media. After autoclaving 10ml broth was suspended in a sterile tube and with inoculating loop bacterial colonies were inoculated into the tube. The tube was incubated for 24 hours at 37 °C. After 24 hours change in colour was examined as an indicator for positive results.

Red color: Lactose Negative	**Yellow color: Lactose Positive**

3.13 Screening of Probiotic bacteria

3.13.1 Screening for Bile Salt Hydrolase Activity

TSA media was prepared for screening the intestinal isolates for bile salt hydrolase enzyme. After autoclaving it was allowed to cool down until the temperature reaches 54 °C and augmented with 0.5% (w/v) salts of Taurodeoxycholic acid and Glycodeoxycholic acid. This media was then poured onto the petri plates. Bacterial strains were streaked on these plates and then incubated at 37°C. Precipitation was observed after 5 days. (Kumar *et al.* 2011).

3.13.2 Screening for Lipolytic Activity

Tween 80 media was used for screening of Lipolytic activity of probiotics as explained by (Serria, 1975). Tween 80 media (lipid source) was autoclaved separately at 15lb pressure for 15 minutes. Then autoclaved TSA (1/100 ml) was added to Tween 80 media. This amalgam was poured onto the petri plates and streaked with intestinal isolates. The plates were incubated for 24 – 72 hours. Changed in colour indicates positive results.

3.13.3 Screening for Proteolytic Activity

For assessing proteolytic activity of probiotic bacteria, Milk agar plates were prepared. 100 ml distilled water was taken and 2.5g agar was added and then autoclaved. After autoclaving the media was set to cool until 50 – 55 °C. Then 10 grams' skim milk powder were added and then plated. Surface dried Milk Agar plates were then streaked and were incubated at 37 °C for 24 – 48 hours. After incubation, for positive results clear zones around the colonies were observed.

3.13.4 Screening for Amylolytic Activity

For performing amylolytic activity, starch agar plates were used. Starch (1g) and nutrient agar (1g) were added to 100ml distilled water and were autoclaved. Selected probiotic isolates were streaked lined on the dried surfaced starch agar plates. Plates were incubated at 37 °C for 24 hours. After incubation, the plates were swamped with 1% of gram's iodine. Clear zones around the streaked lines were observed for positive results.

3.13.5 Screening for Cellulolytic Activity

Intestinal isolates were inoculated on CMC agar media for screening of cellulolytic enzymes activity. The media was prepared as explained by Hankin and Anagnostakis (Hankin and Anagnostakis 1977). The culture was inoculated, incubated at 37 °C till five days. After incubation, 0.1 % Congo red were spread over the plate and stained for 30 minutes. The stained plates were washed thoroughly with distilled water. Then one molar of NaCl was poured onto the plates and left for 5 minutes. Zones were detected for positive results.

Chapter 4

Results

4 Results

4.1 Effect of Energy Restriction (Ramadan fasting) on Human Female Physiology

4.1.1 Effect of Ramadan Fasting on Anthropometric Values

Overweight and obesity are initial signs for all the metabolic diseases. Body Mass Index (BMI) is a measurement of overweight and obesity (Swanton, Frost, & Maryon-Davis, 2007). Age, height and weight of all the participants were recorded before Ramadan and then before Eid i-e. from 25th to 27th Ramadan. It was observed that at the end of the study there were change in weight and BMI values of the participants. Weight of F4 decreased from 94 to 90 Kg, F5 decreased from 115-110 Kg, F7 was reduced from 62 to 59 Kg. Wight was also reduced F11, F14 and F25.whereas weight of F23 increased from 55 Kg to 58 Kg. Sample F3, F4, F7, F14 and F25 showed decrease in BMI value while F23 showed increase in BMI values. When average of all the Weight and BMI values were taken. Weight and BMI both were decreased at the end of the study as shown in figure below. Table 4.1 shows anthropometric values of all the individuals before Ramadan and after the Ramadan.

Table 4.1: Anthropometric Parameters before and at the end of Study

Sample I.D	Age	Height (Inc)	Weight (Kg) before Ramadan	Weight at the End of study	BMI before Ramadan	BMI at end of study	BMI Status/After
F1	25	64	52	52	19.7	19.5	Normal
F2	23	62	42	42	16.94	16.94	Underweight
F3	**35**	**64**	**94**	**90**	**35.5**	**34.1**	**Obese II**
F4	**30**	**68**	**115**	**110**	**38.5**	**36**	**Obese II**
F6	24	63	53	53	20.7	20.7	Normal
F7	**23**	**62**	**62**	**59**	**25**	**23**	**Overweight/ Normal**
F9	24	63	52	52	20.4	20.4	Normal
F11	25	64	55	54	21	20.8	Normal
F12	25	65	57	57	21.6	21.6	Normal
F14	25	62	62	58	25.0	23.4	Normal
F20	24	64	54	54	21.1	21.1	Normal
F23	**23**	**63**	**55**	**58**	**22.3**	**22.7**	**Normal**
F25	22	62	44	40	18.3	16.7	Underweight
F29	34	63	45	45	17.6	17.6	underweight

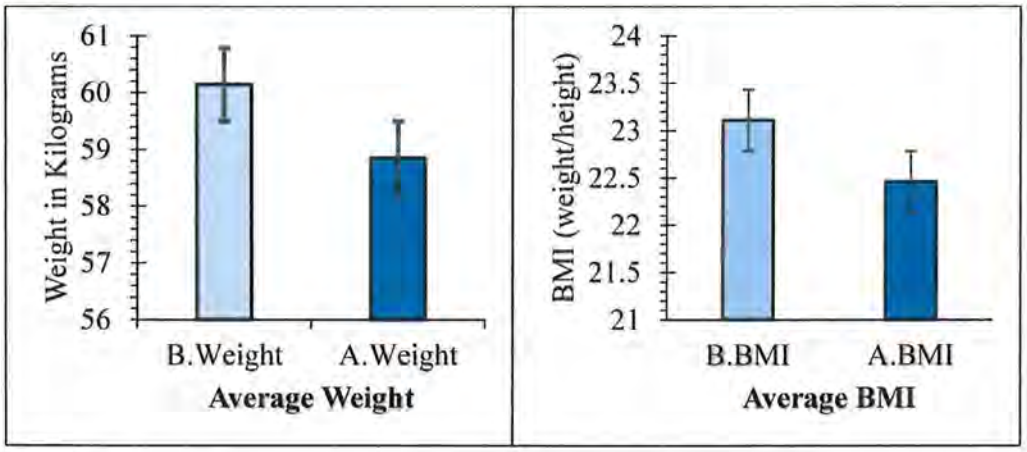


Figure 4.1: Showing mean changes in Anthropometric and BMI Values before and at the end of the study

4.1.2 Effect of Ramadan Fasting on Blood Parameters

Blood was analyzed and different parameters were recorded before and the end of the Ramadan. Blood was analyzed for "Total Cholesterol (TC)", "Total Triglycerides (TG)", "High Density Lipoprotein (HDL)", "Low Density Lipoprotein (LDL)" and "very Low Density Lipoprotein (vLDL)". It was observed that blood parameters have been changed after collecting samples before Eid. Almost all the samples had change in their blood parameters. HDL was increased in all samples except F3. TC was decreased in all samples except F2, F11, F23 and F25. TG was decreased in F2, F12, F23 and F25. LDL decreased in all the samples except F2, F11 and F25. Very LDL was decreased in the samples in which total cholesterol and triglycerides were decreased. Significant changes were observed when mean of all samples were taken. Mean total cholesterol decreased from 152mg/Dl - 141mg/dL. Mean total triglycerides decreased from 104mg/dL to 100mg/dL. Similarly, mean low density lipoproteins decreased from 104 to 93mg/dL. Very low density lipoprotein decreased from 21 to 19mg/dL whereas Mean HDL increased from 27 to 28mg/dL. Table 4.2 shows results of all the lipid profile for all the samples. Figure (1) shows mean values of TC, (2) shows mean values of TG, (3) shows mean values of HDL (4) shows mean values of LDL and (5) shows mean values of vLDL of all the samples before Ramadan and at the end of the study.

Table 4.2: Lipid Profile values before and after Ramadan

Sample ID	Before TC (mg/dL)	After TC (mg/dL)	before TG (mg/dL)	After TG (mg/dL)	Before LDL (mg/dL)	After LDL (mg/dL)	Before HDL (mg/dL)	After HDL (mg/dL)	Before vLDL (mg/dL)	After vLDL (mg/dL)
F1	191	184	68	66	139.4	131.8	38	39	13.20	13.60
F2	130	140	95	102	85	90.6	26	29	19.0	20.4
F3	200	149	189	141	120.2	88.8	42	32	37.8	27.4
F4	170	118	160	88	106	53.4	32	47	32.0	17.6
F6	118	107	98	92	75.4	64.6	23	24	19.6	18.2
F7	160	137	93	84	174.4	105.2	11	15	19.8	16.8
F9	147	145	107	105	103.6	102	22	22	21.4	21.0
F11	145	152	98	93	99.4	105.4	26	28	19.6	19.8
F12	108	102	62	63	73.6	66.4	22	23	12.4	12.6
F14	144	144	115	95	83	70	38	55	23.0	19.0
F20	272	199	115	100	207	136	42	43	23.0	20.0
F23	74	100	55	93	43	24.4	20	57	11.0	18.4
F25	176	201	95	107	122	143.6	35	36	19.0	21.0
F29	184	169	103	98	131.4	115.4	32	34	20.6	19.0

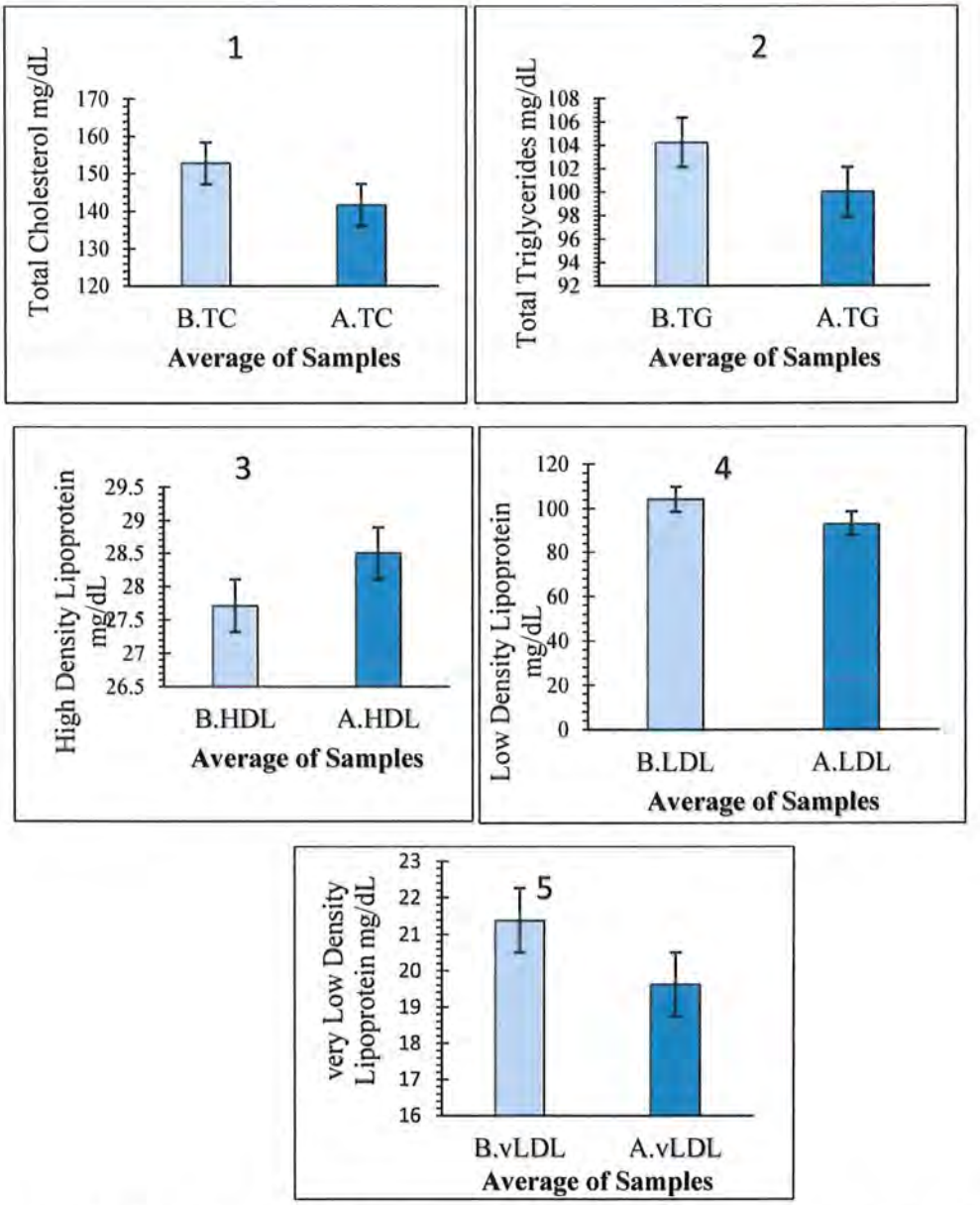


Figure 4.2: (1) shows mean changes in TC, (2) shows mean changes in TG (3) shows changes in HDL (4) Mean of LDL and (5) Mean of vLDL at the end of Study

4.2 Effect of Ramadan Fasting on Gut Microbiome

4.2.1 Evaluation of Ramadan Effects on Gut Microbiome via Culturing Method

4.2.1.1 Macroscopic Examination

Following incubation period for 24 to 48 hours, culture plates were observed for growth of intestinal microbiota. Colony morphology of growth media for all samples were observed on all the five media which are: BHIA, M17, MRSA, MacConkey and OGA. Colonies on BHIA media plates were off white or pale yellow, irregular, circular and of different sizes ranging from minute to small and large having either entire or undulate margins. The colonies on M17 agar plates were pale yellow and white. The morphology of colonies was circular, evenly margined and the size range from minute to large. The MRSA plates had pin pointed, small colonies having pale, white and off white colours while MacConkey plates were having lactose positive as well as lactose negative colonies of various sizes (minute, small and big). Some colonies on MacConkey plates were having rough margins while some were having smooth margins. Finally, OGA media plates showed mostly small whitish colonies of uniform sizes. However, some plates had fluffy white wool like colonies.

Table 4.3: Colony Morphology on Media Plates

Media (Organism's type)	Colony Shape	Colony Size	Colony Color	Colony Margins	Colony Surface
TSA (Total Bacterial Count)	Circular/ irregular	Minute Small Large	Milky White Pale	Entire Undulate	Raised Flat
M17 ***(Enterococcus/Streptococcus)***	Circular	Pin pointed Small Large	Pale Yellow White	Entire Smooth	Flat
MRS (Lactic Acid Bacteria)	Circular	Pin pointed Small	Of white Pale	Entire Smooth	Flat
MacConkey (Enteric G-ive bacteria)	Circular	Minute Small Large	Yellow Pink	Entire Undulate	Flat Raised
OGA (Yeasts)	Circular	Small	White	Entire	Fluffy Convex

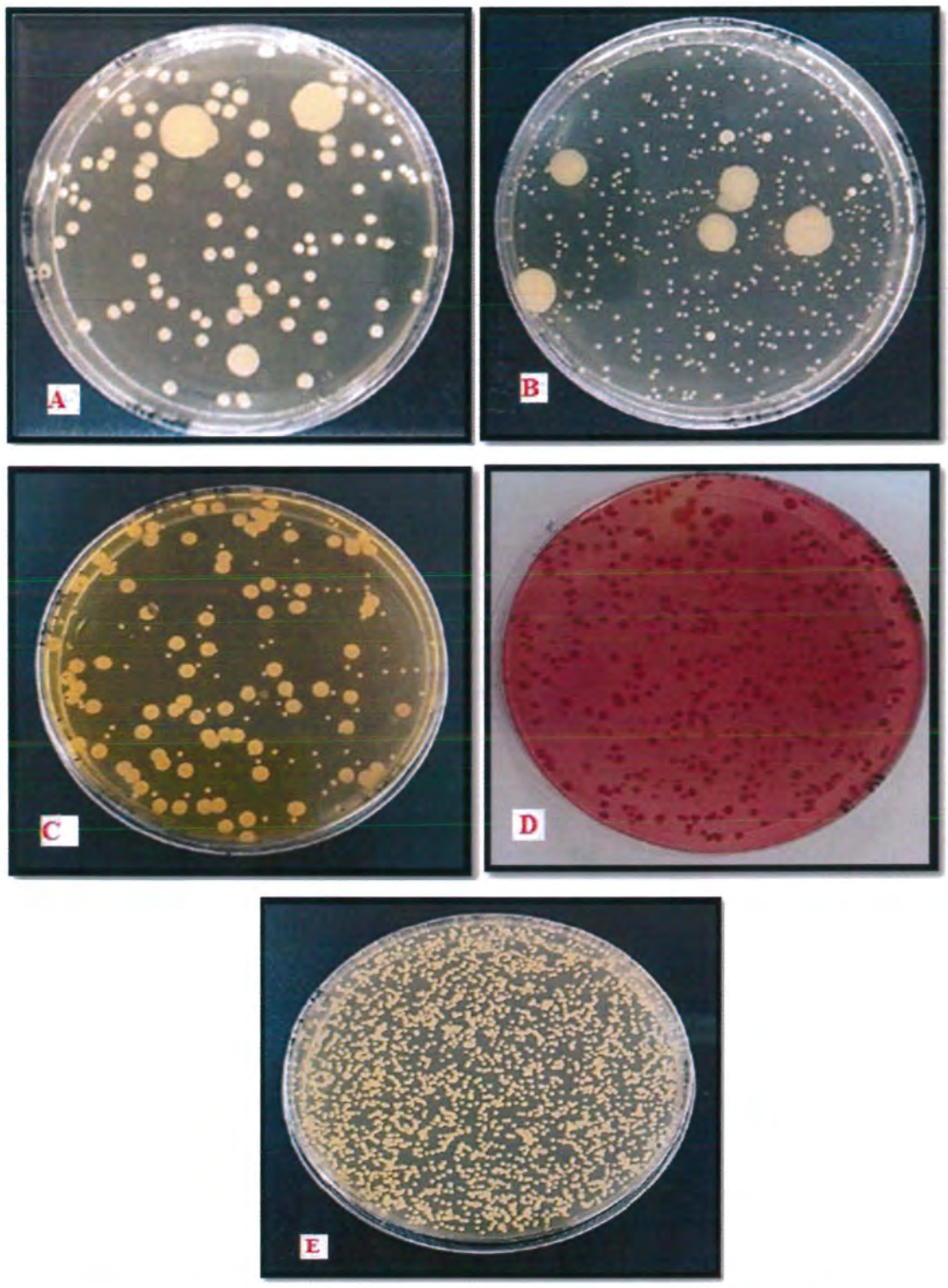


Figure 4.3: (A) and (B) shows colonies on BHIA and M17, (C) and (D) shows colonies on MRSA and MacConkey, and (E) Colonies on OGA plate.

4.2.1.2 Microscopic Examination

Isolated colonies from media were taken, slides were prepared and stained with Gram stain and lacto phenol cotton blue stain. Air dried slides were observed under microscope using emersion oil. BHIA plates contained gram negative bacilli and gram positive cocci and bacilli both. Colonies on M17 agar plates were gram positive when were seen under microscope. MRS colonies were gram positive bacilli. Colonies on MacConkey agar plates were identified gram negative bacilli. Lacto phenol blue strain was used detect colonies on OGA media for yeast.

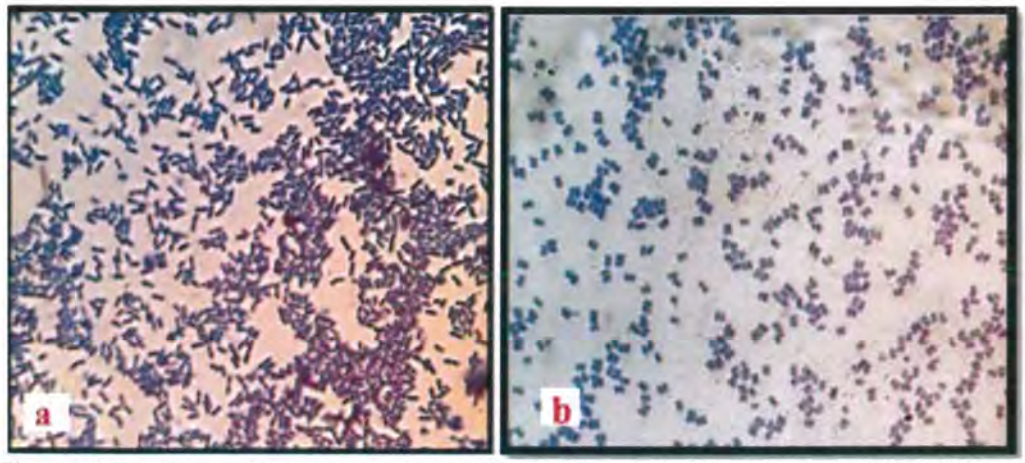


Figure 4.4: shows staining results (a) shows Gram Positive *Lactobacilli* (b) Gram positive *Lactococci*

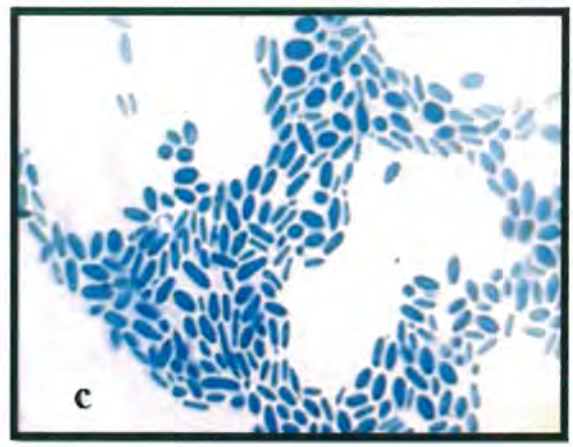


Figure 4.4: (c)Yeast

4.2.1.3 Colony Forming Unit Count (CFU/Gram)

Colony forming units were summed by colony counter for purpose of influence of Ramadan fasting on the human female gut floral assortment. Five different media were used and colonies were counted on each plate. This was an irregular method to guess the expanse and kind of microorganisms existing earlier and later the study in the female gut.

On BHIA total bacterial count was noted between 2.9 x 10^8 and 2.9 x 10^{10} CFU/g. On M17 media plates Streptococcus and Enterococcus were counted from 2.91 x 10^8 to 3.58 x 10^9 colonies per gram of sample. Similarly, estimated number of Lactic Acid Bacteria on MRS agar plates was documented between 4.58 x 10^3 to 8.82 x 10^5 colonies/g.

Number of Gram negative enteric bacteria on MacConkey media was recorded between 0 to 2.0 x 10^7 CFU/g. Yeasts were counted 1.0 x 10^1 – 8.90 x 10^5 CFU/g on OGA media plates. When mean of all the values was taken, notable changes were observed at the end of the Ramadan.

Average aerobic bacterial count was significantly decreased at the end of the study as shown in Figure 1. Figure 2 shows that Enterobacteria count increased at the end of the study. Figure 3 shows fungal count increased at the end of study. Average lactobacillus count was significantly decreased at the end of the study as shown in Figure 4 while Figure 5 shows increased mean Gram Negative Streptococci and Enterococci count.

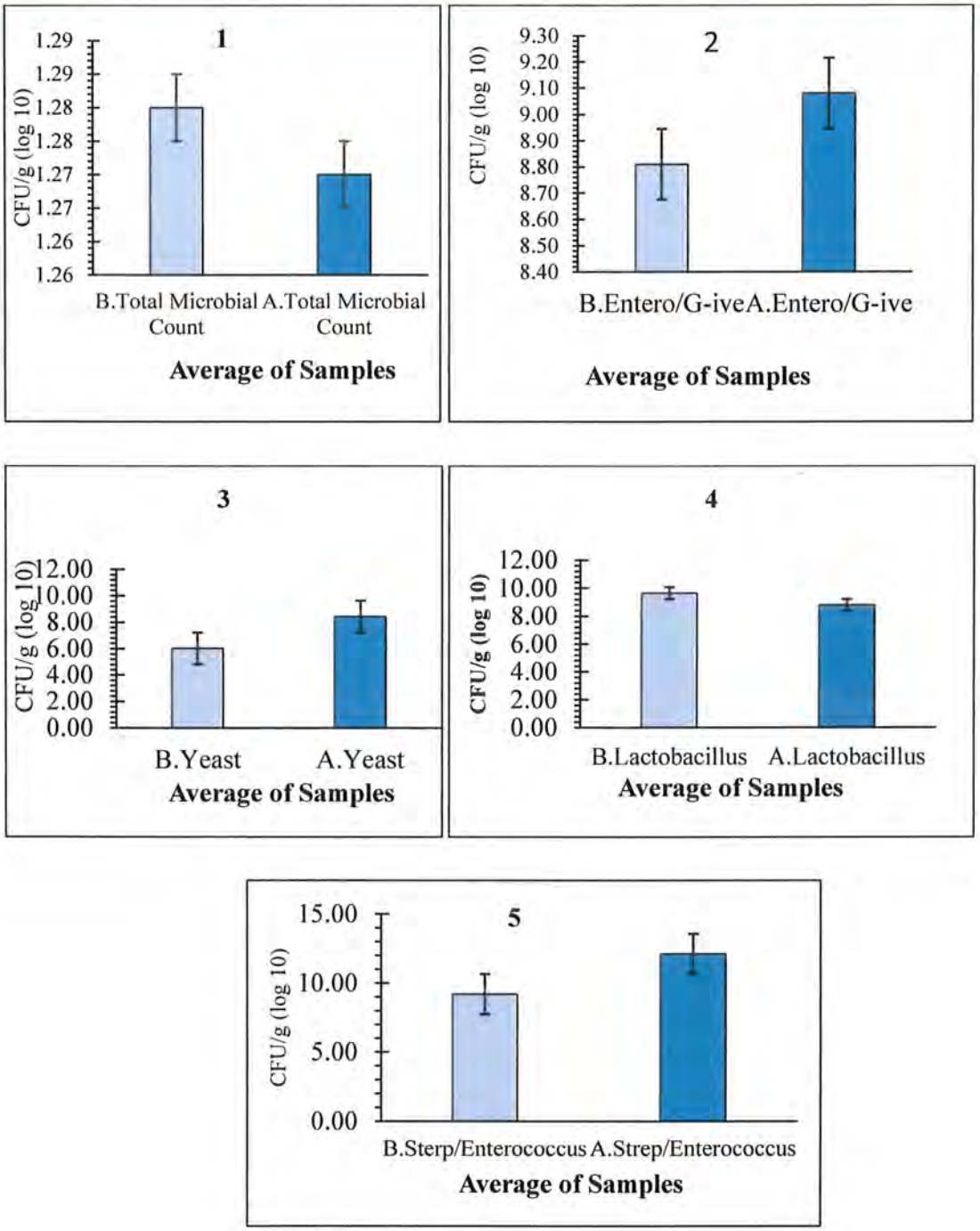


Figure 4.5: Showing mean changes in (1) total aerobic microbial count (2) Gram negative enteric count (3) Fungal count (4) lactobacilli count and (5) shows average streptococcus count at the end of the study.

4.3 Grouping of Participants via Anthropometric, Physiological and CFU Change

Anthropometric values, physiological parameters and gut microbiology was recorded before and at the end of study. A clear change was seen in all parameters when comparison was made with each other. All the samples were grouped together based on these parameters. For selection of samples for metagenomics, clustering was performed using Agglomerative Hierarchical Clustering (AHC) statistical software and distance between different groups were calculated with Pearson Correlation Coefficient ($p < 0.05$). The software showed the graphical presentation of clustering in a dendrogram. The clades in dendrogram divided all the samples into three clusters or groups. F23, F7, F6, F1, F11 and F12 were grouped together. F25, F29 were in same group. F4 and F14 presented a group and F9 were totally different from all the samples. F7 and F23 were taken as representatives for two groups. These representative samples were selected for metagenomic analysis. Figure given below represents all the samples in different similar clusters.

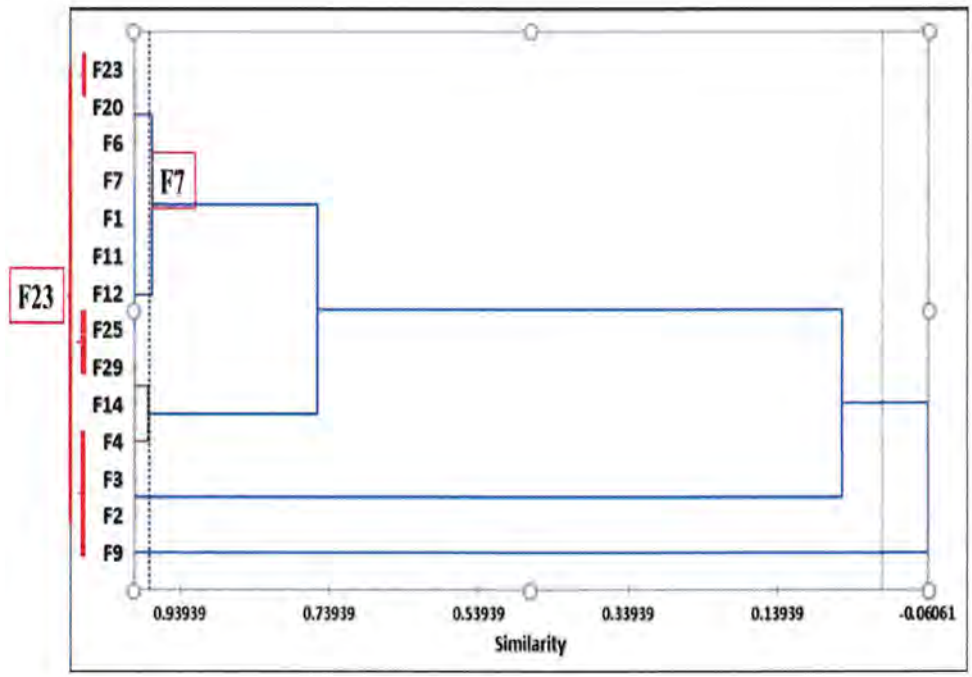
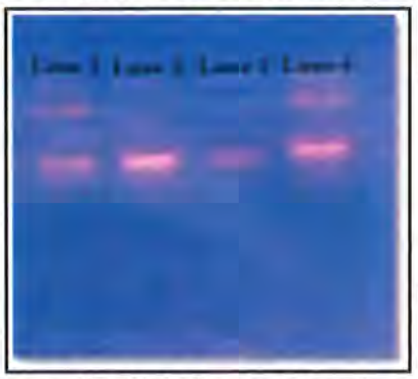


Figure 4.6: Cluster dendrogram

4.4 Evaluation of Ramadan Effect on Human Female Gut Microbiome through Culture Independent Method

4.4.1 Gel Electrophoresis

Gel electrophoresis was performed for conformation of the presence of DNA



Lane 1: F7 I
Lane 2: F7 II
Lane 3: F23 I
Lane 4: F23 II

Figure 4.7: Gel Electrophoresis results

4.4.2 Nanodrop

Quantity and quality of the extracted DNA was checked by nanodrop. The quantity and purity of the extracted met the criteria for metagenomic analysis. F7I showed highest quantity while F7II showed the lowest. Quantity & purity of the samples are given in the table below. The results for nanodrop are shown in a table 4.4.

Table 4.4: Nanodrop Results of Selected Samples

Sample ID	ng/µl	A260	A280	260/280
F7 I	553.60	11.072	5.503	2.01
F7 II	18.46	0.369	0.268	1.38
F23 I	25.03	0.501	0.371	1.38
F23 II	66.23	1.325	1.038	1.28

4.4.3 Metagenomic Analysis

DNA fragments were sequenced and filtered, low quality reads were removed. The remaining reads were analysed computationally and assigned to phylum, genera and species based on similarity. Highest similarity tag was set 97% for assigning the read to species.

Comparative abundance analysis of overall bacterial diversity in the present study contains 365 species. After setting a limit of < 0.1% for comparing bacterial species, 79 species were left for F7 whose weight was decreased at the end of the study and 88 were left for sample F23 who gained weight in Ramadan.

When OUT's were counted, and compared, it was seen bacterial count has decreased to significant level in F7 while for F23 it decreased but at negligible level. Table depicts over all bacterial counts in both the samples.

Table 4.5: Total OTUs Count before and after the Ramadan

Sample ID	Number of OTUs before	Number of OTUs After
F23	389062	250090
F7	262936	261588

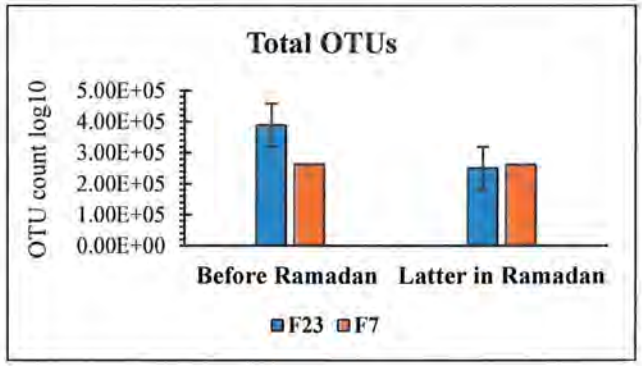


Figure 4.8: Bacterial total count

4.4.4 Diversity Index

Alpha diversity, relative abundance and dominating bacterial species were determined by using OUT's, number of reads, Shannon and Simpson index. The total number of OTU's were high in samples collected before Ramadan as compared to OUT's obtained after Ramadan. It was observed that diversity evenness and richness were improved in sample F7 latter in Ramadan. but no such change was seen in sample F23 at the end of the study. The table and figure shows diversity index of all samples before and after study.

Table 4.6 Diversity Index of all the samples before and at end of the study

Sample ID	Number of OTUs	Number of Reads	Shannon Index	Simpson Index	Observed Species
B. F23	389062	277	3.43	0.48	277
A. F23	250090	276	3.71	0.82	276
B. F7	262936	278	3..36	0.85	278
A. F7	261588	277	3.29	0.94	277

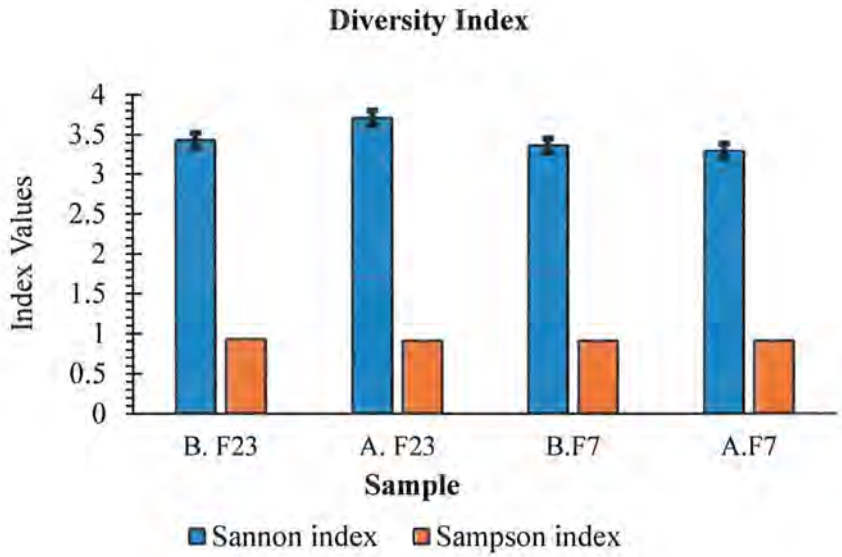


Figure 4.9: Showing Diversity indexes of selected samples before and after the Study

4.5 Changes at Phylum level

Remarkable changes were observed at phylum level. Firmicutes, Bacteriodes, Actinobacteria, Proteobacteria, Cyanobacteria and Spirocheates were the dominating phylum in both the samples. Firmicutes were making the largest part of both the samples with bacteriodes on second number. After Ramadan percentage of Firmicutes and Bacteriodes were decreased in both the sample. Protebacteria were increased in sample F23 but not in sample F7. At species level sample F23 show changes in number as well as type of species before and after the study. Before Ramadan *Roseburia facies* was the highest species making 17% of the total bacterial population which decreased to 1%. *Prevotella copri* was 16% which decreased to 8.6%. *Bifidobacterium Adolescentis* was decreased from 8% to 5.8%. *faecalibacterium* spp. Decrease from 6.09% to 1.9%. *Ruminococcus flavefaciens* from 5% to 2%. While some species were increased e.g. *Shigella sonnei* increased from 0.1% to 16%, *clostridium* spp. from 3% to 14%

Clostridium perfringen, from zero to 10% and *Subdoligranulum* spp. from 1 to 5%. Sample F7 also show changes in species *Clostridium* spp. were reduced from 22% to a negligible amount of 0.09%. *Clostridium disporicum* reduced from 8.6 to 3.2%, *Turicibacter* spp. from 7 to 1%, *Oscillospira* spp. *Ruminiclostridium, Clostridium termitidis* from 4% to 0.1%, some species were increased at the end of the study like *Ornithinibacillus sp.* was increased from 0.01 percent to 24%, *Lachnospira* spp. increased from 0.01 percent to 6% and *lachnospira pectinoschiza* from 0 to 3 percent. Figure shown below shows the percentage of each phylum before and at the end of study.

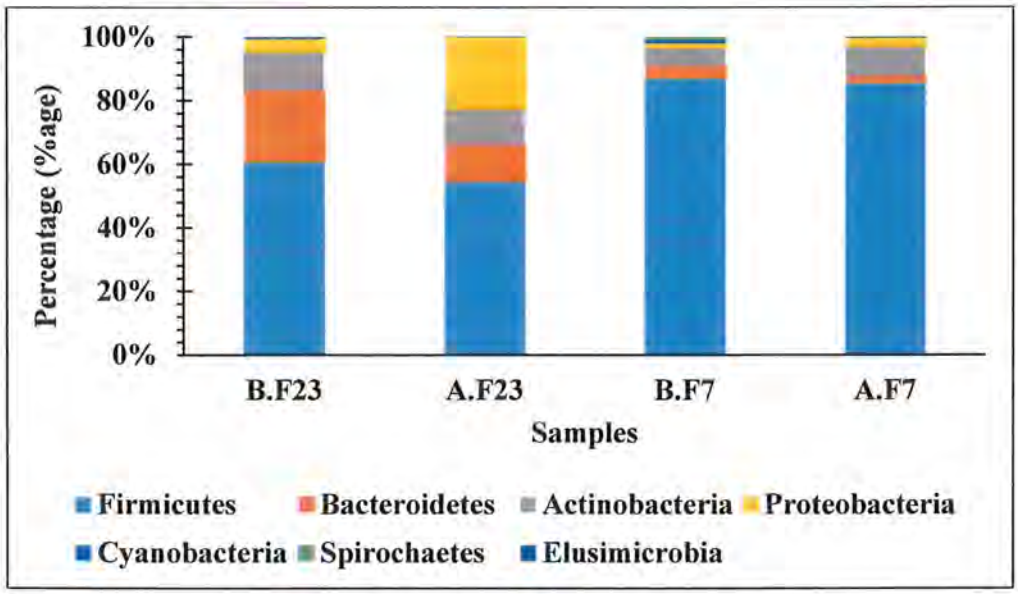


Figure 4.10: Phylum level changes in all samples before and at the end of study

4.6 Changes at Species level

The abundance and types of species were also changed at the end of study in both samples. In sample F7, initially there were total 280 species having 97% similarity index before Ramadan. After omitting species having < 0.1 % relative abundance 68 remained whereas there were total 278 species in F7 group having 97% similarity index at the end of Ramadan. After omitting species having < 0.1 % relative abundance 56 remained. *Clostridium* spp was dominated in F7 group before Ramadan whereas *Ornithinibacillus sp.* were dominated in F7 group after Ramadan. Some of the dominating species in F7 before Ramadan were: *Clostridium* spp (22.99%), *Ruminococcus* spp (8.63%), *Ruminococcus* spp (7.85%), *Turicibacter* spp (7.49%) *and Oscillospira* spp (4.28%). The dominating species after Ramadan were: *Ornithinibacillus sp* (27.84%), *Lachnospira* spp (6.12%), *Ruminococcus* spp (6.06%), *Lachnospira*

pectinoschiza (3.96%) and *Pseudomonas putida* (3.76%). All dominating species are shown in figure below.

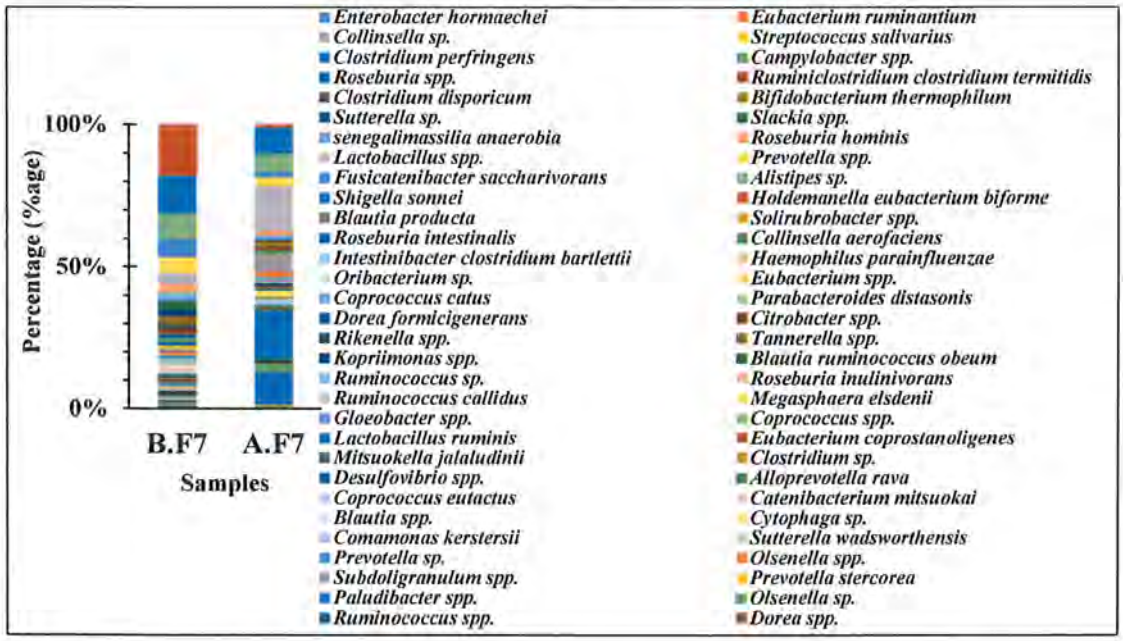


Figure 4.11: Showing percentage of species in F7 before and at the end of study

Similarly, changes were seen in sample F23 at the end of study. There were total 277 species in F23 group having 97% similarity index before Ramadan. After omitting species having < 0.1 % relative abundance 68 remained. *Roseburia faecis* was dominated in F23 control group before Ramadan. The five most occurring species were: *Roseburia faecis* (17.16%), *Prevotella copri* (13.06%), *Bifidobacterium adolescentis* (8.52%), *Faecalibacterium* spp (6.09%) and *Ruminococcus flavefaciens* (6.09%) whereas there were total 276 species in F23 group having 97% similarity index at the end of Ramadan. After omitting species having < 0.1 % relative abundance 52 remained. *Shigella sonnei* were dominated in F23 group after Ramadan. The other dominating species were: *Shigella sonnei* (16.30%), *Clostridium* spp (14.98%), *Clostridium perfringens* (10.71%), *Prevotella copri* (8.61%) and *Subdoligranulum* spp (5.89%). The results are shown in figure 4.12.

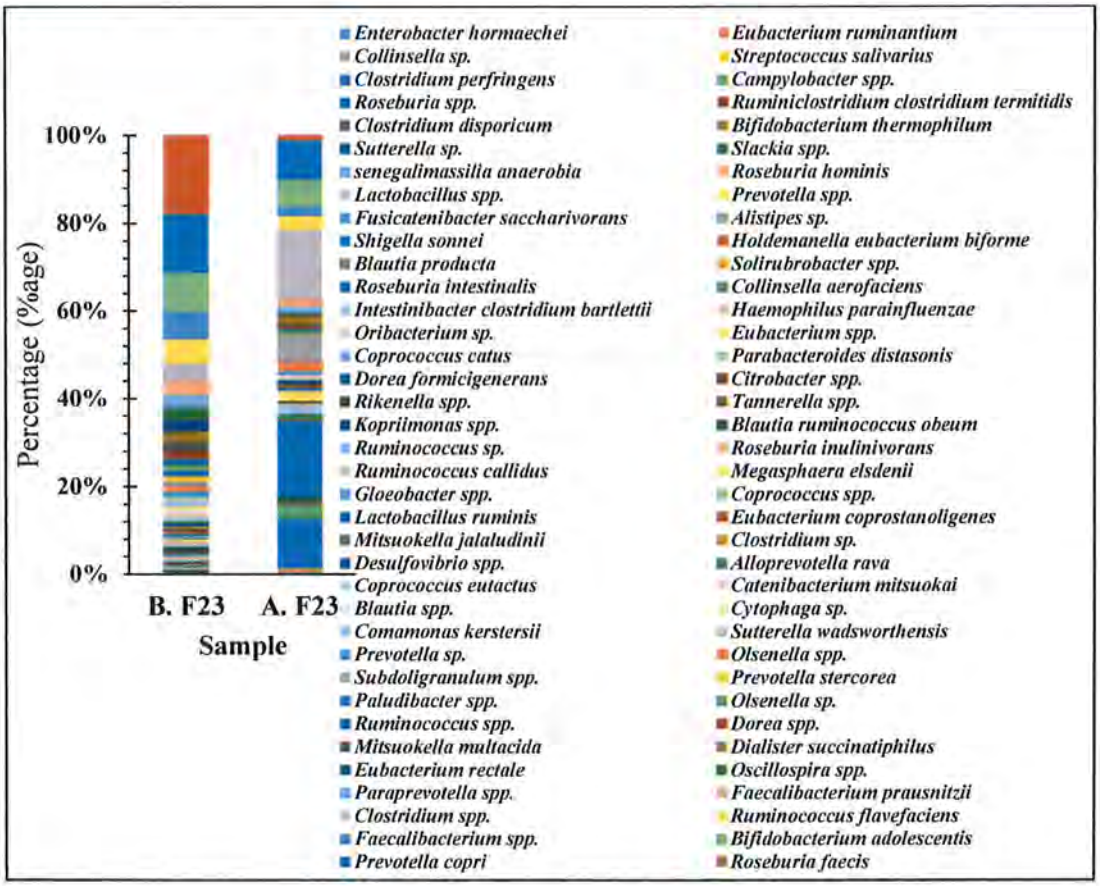


Figure 4.12: Showing percentage of species in F23 before and at the end of study

4.7 Species Level Changes in all Samples

Overall, changes occurred at specie level in both samples at the end of study. To see the changes, limit of 1% was adjusted for all bacterial species. It was observed that some disease-causing species were decreased at the end of study like *Dorea* spp, *Clostridium* spp, *Intestinibacter clostridium bartlettii* were decreased. The table below shows changes in sample and the functions of bacterial species.

Table 4.7: List of Bacterial species with their functions, changed in all groups at the end of the Study

Species	Function	Type of change
Shigella sonnei	Pathogenic	Increased
Subdoligranulum spp.	Wide range of fermentation	Increased
Olsenella spp.	Mouth flora, prevent tooth decay	Increased
Collinsella aerofaciens	Wide range of fermentation (Carbohydrates)	Increased
Dorea spp.	IBD	Decreased
Lactococcus plantarum	Probiotic	Increased
Faecalibacterium spp.	Electron shuttle	Decreased
Dialister succinatiphilus	Propionate producer	Decreased
Faecalibacterium prausnitzii	Electron shuttle	Decreased
Intestinibacter clostridium bartlettii	Human pathogen	Decreased
Paraprevotella spp.	Xylan producing bacteria	Decreased
Roseburia faecis	Probiotic	Decreased
Oscillospira spp.	Unknown	Decreased
Eubacterium coprostanoligenes	Cholesterol reducer	Decreased
Ruminococcus spp.	Cellulose degrader	Decreased
Ruminiclostridium clostridium	Human pathogen	Decreased
Paludibacter spp.	Propionate producer	Decreased

4.8 Comparison of Gut Bacteria with other Published Data

To know the differences and similarity of Pakistani gut bacterial population with other countries, published data was compared. It was observed that Pakistani gut bacterial population resembles with China gut bacterial population.

Table 4.8: Gut Comparison

Country	USA	Korea	Japan	China	India	Pakistan
Dominant Phylum	Firmicutes	Bacteriodes	Actinobacteria	Bacteriodes	Firmicutes	Firmicutes
Dominant Group	Firmicutes Bacteriodes	Bacteriodes Actinobacteria	Bacteriodes Actinobacteria	Firmicutes Proteobacteria	Firmicutes Actinobacteria	Firmicutes Proteobacteria
Reference	Mueller *et al.*, 2006				Dehingia *et al.*, 2015	Present study

4.9 ..Identification and Characterization of Probiotic bacteria from selected fecal samples

For isolation of probiotic strains, colonies were selected from MRSA and M17 agar plates of selected samples. Total of eight strains were isolated from both media. The strains isolated from MRSA plates were labelled as F7i, F7 ii, F23 ii while strains on M17 were labelled as f7i. f7ii, f23i & f23ii.

4.9.1 Microscopic Identification

All selected strains were gram stained before microscopy. MRSA strains were gram positive bacilli whereas strains from M17 were gram positive cocci. The strains appeared purple under microscope which confirms initial recruitment for probiotics bacteria.

4.9.2 Biochemical Identification

For biochemical identification, strains from MRSA and M17 were selected. These samples were investigated for catalase, oxidase and lactose fermentation.

4.9.3 Catalase Test

Catalase test is performed to detect the probiotic strains as all probiotics are catalase. No bubble formation occurred for all the MRSA and M17 isolated strains, fulfilling a criterion for selection of probiotics.

Figure 4.9.13: F23i & F23ii (catalase -) and F7i & F7ii (catalase -) on M17

Figure 4.9.14: F23i & F23ii (catalase -) and F7i & F7ii (catalase -) on MRS

4.9.4 Oxidase Test

Change in filter paper colours indicates positive results. All four strains on M17 showed negative result for oxidase test which is a property of probiotics as shown in figure below.

Figure 4.9.15: Left figure shows F23i and F23ii (oxidase-) on M17 while right shows F23i and F23ii (oxidase -) on MRS

4.9.5 Lactose Utilization Test

Probiotic ferment Lactose and form lactic acid lowering pH of the media. This test was performed for all the isolated strains. All the strains transformed the red color media into yellow color after incubation showing positive results as shown in figure.

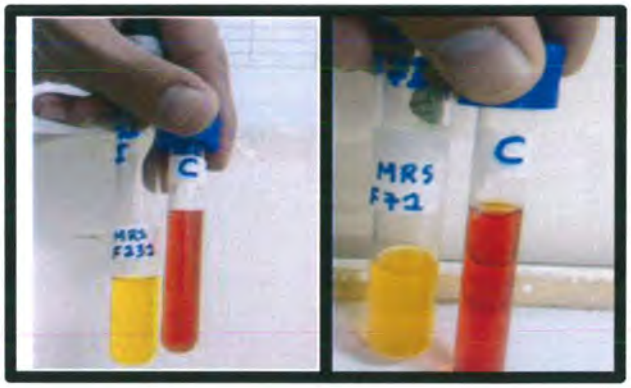


Figure 4.9.16: F23i (positive) and F7i (positive) on MRS

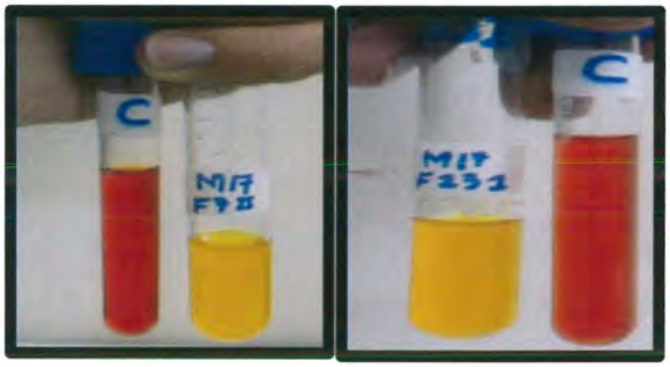


Figure 4.9.17: F7ii (positive) and F723ii (positive)

4.10 Screening Tests for Probiotics

4.10.1 Screening for BSH Activity

TSA media was used to grow isolates supplemented with Bile salts. After incubation period of 5 days at 37°C, some strains showed BSH activity.

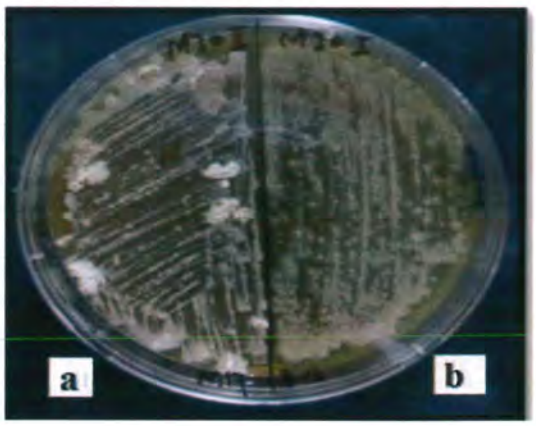


Figure 4.10.18: shows (a) BSH Positive and (b) BSH Negative test

4.10.2 Screening for Lipolytic Activity

Tween 80 was used as a lipid source TSA was supplemented with Tween 80 along with phenol red as an indicator for screening for Lipolytic activity. The media was incubated for two to three days at 37 °C. Change in colour indicates positive results.

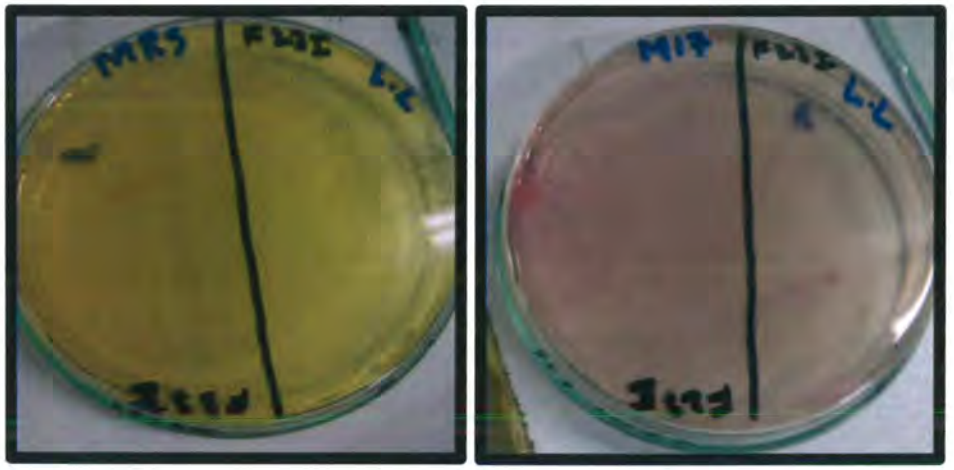


Figure 4.10.19: Lipolytic Positive and Lipolytic Negative test

4.10.3 Screening for Proteolytic Activity

Milk agar media was used for screening of proteolytic activity. Clear zones were observed around the colonies on Milk agar media.

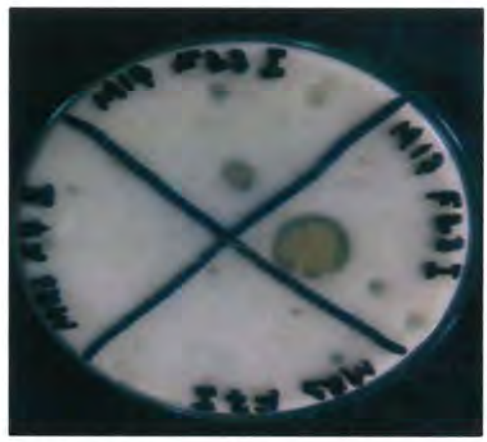


Figure 4.10.20: Proteolytic Positive test on sample F23i

4.10.4 Screening for Amylolytic Activity

Starch media was used for screening of amylolytic activity. Selected isolates were incubated at 37 °C for two days. Clear zones were seen that indicates positive results.

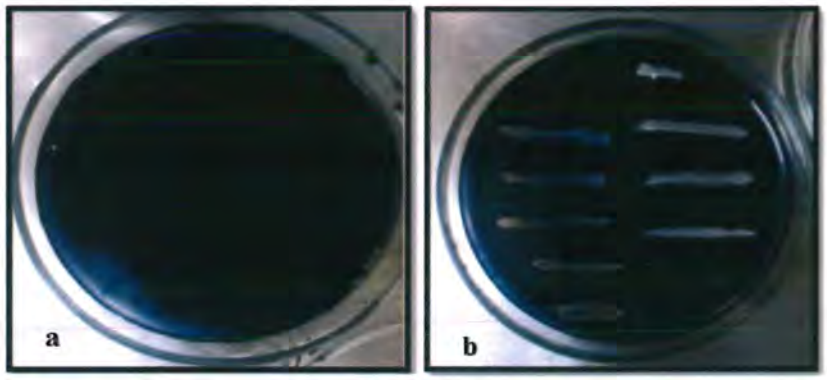


Figure 4.10.21: (a) Amylolytic Negative and (b) Amylolytic Positive tests

4.10.5 Screening for Cellulolytic Activity

CMC media supplemented with NaCl Congo Red was used for screening of cellulolytic activity. Strains were positive when clear zones were seen when washed with NaCl. The figure shows positive results for cellulolytic activity.

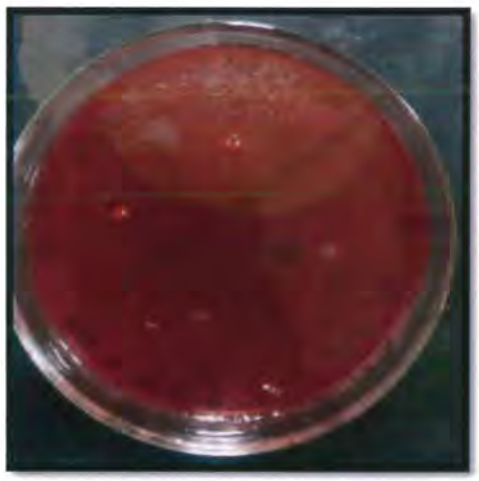


Figure 4.10.22: Cellulolytic Positive Results

4.11 Biochemical Characterization Results for all Strains

Samples F23ii, f23iii, f23iv and F7i showed maximum tested probiotic properties and remaining three samples showed minimum probiotic properties while one sample **F23i** showed all the tested probiotic properties. The table below shows biochemical characterization of all strains.

Table 4.10.9: Biochemical and Enzymatic Characterization Results of Intestinal Isolates

Strain id	Catalase	Oxidase	BSH	Lipolytic	Proteolytic	Amylolytic	Cellulolytic
F23i	Negative	Negative	Positive	Positive	Positive	Positive	Positive
F23ii	Negative	Negative	Positive	Negative	Positive	Positive	Positive
f23iii	Negative	Negative	Positive	Positive	Positive	Negative	Positive
f23iv	Negative	Negative	Positive	Positive	Positive	Negative	Positive
F7i	Negative	Negative	Positive	Negative	Positive	Positive	Positive
F7ii	Negative	Negative	Positive	Negative	Negative	Positive	Positive
f7iii	Negative	Negative	Negative	Positive	Positive	Negative	Positive
f7iiv	Negative	*Negative*	Negative	Negative	Positive	Negative	Positive

Chapter 5

Discussion

5 Discussion

Human body is a host of tremendous microorganisms. Most of these microbial cells reside in human gut. It is reported that human gut is colonized by 10^{14} microorganisms (Clark & Coopersmith, 2007). The number of these microbes are ten fold more than the human somatic cells. These microorganisms are collectively known as Gut Microbiota and their genome is known as Gut Microbiome. The gut microbiota encodes 9×10^7 unique genes whose product perform more than a host's genomic products (Yang *et al.*, 2009). It is considered that a huge number of microorganisms belong to bacterial cells, comprising of 1000 species with Firmicutes and Bacteriodes - the most dominant groups (Honda & Takeda, 2009). All healthy individuals contain specific and balanced gut microbiota under normal circumstances. This flora remains stable over large time and their collective genome can be used as alternative to finger print. Studies have reported a direct relationship between intestinal microorganisms and human health. It is well established that balanced gut microbiota is crucial for maintaining host physiology and health (Jandhyala *et al.*, 2015). The normal and balanced condition of the gut bacteria is called orthrobiosis. However, under some conditions an imbalance of gut microbiota occurs which is termed as dysbiosis. The dysbiosis of gut has been associated with variety of diseases like obesity, allergies, cancers, autoimmune diseases and many other metabolic disorders. The initial contributors for metabolic diseases are obesity and over weight (Vuksan *et al.*, 2016). Over the past years, research is more focused on gut modulation for managing and controlling gut associated diseases like obesity etc. Various methodologies have been employed for gut modulation. Some of the main methodologies includes modulation by antibiotics, synbiotics, prebiotics, probiotics, weight-loss surgeries and faecal transplantation (Cueva *et al.*, 2017). "Eat less live better and longer" is another suggested way for curing all the chronic diseases. Weight loss therapies have proved to be effective and efficient for managing metabolic disorders. They relieve depression, improve cardiovascular status, reduce obstructive sleep apnea etc. (Scheen, 2017).

Energy Restriction (Fasting) is type of weight loss therapy that is being used since ancient times. Researches conducted have shown that energy restriction results in weight loss and ameliorated blood profiles. An extensive literature is present to support health implications of Ramadan fasting (Aloui *et al.*, 2016). Ramadan imparts positive impacts on inflammatory diseases, sleepiness patterns and mood swings etc. It is also proposed that type of the diet and time in between the meals have impact on gut microbiota and human health (Leser & Mølbak, 2009).

Therefore, the current study aims to conducted the impact of the Energy Restriction (Ramadan fasting) on human female physiology and human gut microbiome. The purpose of our study was to rationalize the association of Ramadan fasting on gut microbiota. The objective of study consists of evaluating the impact of Ramadan fasting on gut microbiota. The study also intends to find if Ramadan enhances beneficial bacteria or decreases bad bacteria. For this purpose, blood and faecal samples were collected twice before and after the Ramadan. The samples were collected from thirteen volunteers of age ranging from 22 to 35 years who were fasting for the entire month of Ramadan. Weights of participants were recorded before Ramadan and at the end of Ramadan. It was observed that 1 to 5 Kgs were reduced at the end of study. These results are inconsistent with the previous findings where it was concluded that on average, 2-3 Kg weight gets reduced during the month of Ramadan (Hallak and Nomani 1988, Aloui *et al.*, 2016). Surprisingly, few volunteers also gained weight. Reasons for gaining weight is use of energy dense diets and no exercise or physical activities in Ramadan. (Savitri *et al.*, 2016). Another review also supports same phenomena that intake of energy rich dense foods with no physical activities is the reason for weight gain (Sadeghirad, *et al.*, 2014). Consistent with weight loss, it was observed that lipid profile of the all the volunteers was also improved. It was seen that "Total Cholesterol (TC)", "Total Triglycerides (TG)", "Low Density Lipoproteins (LDL)" and "very low Density Lipoproteins (vLDL)" were decreased at the end of study whereas "High Density Lipoprotein (HDL)" was increased at the end which is a good lipoprotein. These results are supported by extensive literature studies that showed similar results (Abdelgadir *et al.*, 2015; Ara *et al.*, 2016; Antoni *et al.*, 2017). Abdelgadir and co-workers in 2015 conducted a study on three different nations and it was seen that people using normal diets with physical activities have improved lipid profile after Ramadan while people eating more or energy rich diet or doing no or limited physical activities have no change in their lipid profile at the end of Ramadan (Abdelgadir *et al.*, 2015). Yeoh et al, performed a study in 2015 on 29 volunteers in Singapore and at the end of the study he concluded that weight was reduced and lipid profile was also improved in volunteers who were taking normal diets routine diets and doing physical activities (Yeoh *et al.*, 2015).

The study presented that the total bacterial number was reduced at the end of Ramadan which is a supporting evidence suggesting the impact of Ramadan on gut microbiota. Before Ramadan, the samples had more bacterial richness whereas at the end of Ramadan the richness was decreased and diversity was increased. These findings have been previously reported in a study conducted by Mokkala et al. They inspected overweight females had high bacterial

richness that caused high serum zonulin and leaky gut (Mokkala *et al.*, 2016). Another research concluded that increased richness is linked with parasitic gut (Andersen *et al.*, 2016). The decrease in bactereial richness is positively correlated with intestinal bacteria. Maine and Kelly explained that increased intestinal bacterial diversity is good for intestinal flora. Increased diversity means there is more potential to degrade and metabolize vaiety of nutrients in gut (Maine & Kelly 2016). Before Ramadan bacterial diversity was decreased in F7 sample but at the end of the study it was increased showing that gut is modulated positively.

In a current study, the bacterial evenness was lowered (0.4 - 0.8 per Simpson's Index) before Ramadan whereas it was increased at the end of Ramadan from 0.8 - 0.9. The results are like a study done by Tito *et al.* They studied the impacts on bacterial richness and reported that decreased richness is associated with chronic inflammation (Tito *et al.*, 2017). Anti-inflammatory bacteria like *Bifidobacterium* were increased in sample F7. Gioielli et al find out that inulin is the key component that favourably increase the number *bifidobacterial* spp (de Souza *et al.*, 2017). It can be assumed that the participant consumed insulin rich diet. These findings are consistent with our results as all samples had *Lactobacillus* spp as gut residents that play pivotal role in lipid metabolism (Tamarit *et al.*, 2016).

The overall bacterial profile of sample F7 was improved. Before Ramadan it had highest percentage of *Clostridium* species whereas at the end of study it had highest percentage of *Ornithinibacillus sp.* The *clostridium* spp. was reduced from 22% to a negligible amount of 0.09%. *Clostridium disporicum* reduced from 8.6 to 3.2%. F7 before Ramadan, carried high Firmicutes then Bacteriodes and low proteobacteria. At the end of the study, Firmicutes and Bacteriodes were reduced and proteobacteria was increased up to 11%. While in sample F23 no significant changes were seen. Shin et al, conduct a study to investigate dysbiosis and diseases by metagenomics. He found and concluded that too high and too low *Proteobacteria* are signs of dysbiosis. A minor percentage of Proteobacteria (<12%) is known healthy one (Shin, Whon, & Bae, 2015).

The sample F23 showed unusual pattern of bacterial population at the end of study. Before the study it had highest percentage of *Roseburia faecis* (17.6 %) and *Prevotella copri* (13%) while at the end of study the dominating group was *Shigella sonnei* (16 %) and *Clostridium* spp (14%). Both species are human pathogens and their source is raw meat and poultry. One source of *Shigella sonnei* is also drinking contaminated water (Thompson & Baker, 2015). There could be many reasons for increased *Shigella sonnei* and *Clostridium* species in human faecal. One

possible reason might be that participant did not take hygienic food and water. As the participant was hostel resident and the diet pattern showed that she mostly ate meat throughout the Ramadan. Other possible cause many be the shredding off mechanism of these pathogens by human commensal bacteria, mucosal layer and epithelial lining of intestinal gut. The commensal bacteria and mucosal layers might have prevented colonization of *Shigella sonnei* (Anderson *et al.*, 2016). The decrease in *Bifidibacterium* at the end of study may be associated with increase weight. Increased *Roseburia* spp are associated with gall stone formation (Tamanai-Shacoori *et al.*, 2017). While increased *Prevotella copri* are associated with auto immune diseases (Campbell, 2014).

Chapter 6

Conclusion

6 Conclusion

Intestinal flora is the key component having direct as well as indirect influence on human health and physiology. Healthy gut flora is often associated with good health while dysbiosis is linked with variety of diseases. Research has been focused to modulate gut flora for curing and management of chronic disease. Various ways are tried for modulation of the gut and still researches investigate a safe and cheap way to modulate gut flora. Diet based therapies are proposed safe one. Ramadan fasting is extensively studied diet based therapy for different kind of diseases and disorders. Ramadan fasting improves blood profile and reduces body weight thus improving overall physiology of the humans. It might have role in modulation of the gut microbiota.

In the present study impact of Ramadan fasting on human physiology and gut microbiome was investigated by collecting samples from nor-mal healthy individuals who fast for 30 days of Ramadan. it was observed that Ramadan have impact on the physiology of the humans such as weight loss or gain and changes blood parameters. Impact on the gut microbiome was checked by culture dependent and culture independent methods. It was observed that positive impact of physiology was associated with positive change in gut microbiota. Weight loss was associated with increased bacterial diversity and evenness. While weight gain was linked with decreased bacterial diversity and reduced evenness and the change was host specific.

Chapter 7

Future Perspectives

7 Future Perspectives

Human gut microflora has been studied extensively over the past two decades. Structure, function and diversity of gut microbiota has been explored. Methods for modulation of gut microbiota for management of diseases have been investigated and suggested. Similarly, Energy restriction and Ramadan fasting have been investigated broadly. They have been linked with heath and disease. But impact of energy restriction and Ramadan fasting on gut microbiota has limited number of publications. This fields needs much more to be explored. In future effect of Ramadan fasting on gut microbiota can be studied on whole metagenomic level, transcriptomic level as well as at proteomic level for better understanding of the effects at functional level (genes, proteins). Similarly, diet specific intervention can also be carried out to know which diet is more efficient in terms of modulation. Effects can be studied at various research populations like diseased and normal ones, samples of specific diseases or disorders. Molecular mechanism how Ramadan effects can be studied in further studies. The gut microbiota is a vital metabolic organ. However, not much is known about how an individual's microbial species interact, establish a dominating position, and react to variations in environmental factors such as diet.

Chapter 8

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8 References

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