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Sensitivity Evaluation of *Lactobacillus* Species Isolated from Diabetic and Non-Diabetic People Against Anti-Diabetic Drugs

by

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Dedicated to Allah Almighty, the most beneficent and merciful, Hazrat Muhammad (S.A. W. W) and my father Amjad Hussain. My mother prayers have always enlightened my way throughout my life. It's also dedicated my work to my brothers M. Khayyam , M. Inaam and M. Ehtisham and all other family members who supported me and taught me that the best kind of knowledge is that which is learnt for its own sake. They taught me that even the toughest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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(Mehwish Amjad)

Abstract

Metabolic diseases including obesity and diabetes have become a social problem for countries in all over the world. Type 2 diabetes mellitus (T2DM) is more widespread and common type of diabetes. In type 2 diabetic patients take oral medication or insulin to maintain blood glucose level. Human gut microbiota has proven to be associated with various metabolic syndromes such as T2D. Most of treatments that are currently in use to treat T2D, especially pharmaceutical have focused on agents that directly influence the signaling pathways and regulate the glucose, now with more understanding of the root causes of T2D suggests that the human gut microbiome targeting would be more appropriate approach for treating T2D. After the ethical approval 50 stool samples of each diabetic and non-diabetic people were collected and processed. Firstly the stool samples were stored in PBS for further use. Then those samples were cultured on nutrient agar. 16S ribosomal RNA sequencing was performed. The phylogenetic analysis was done. In current study the difference of gut microbiota present in diabetic and non-diabetic individuals was investigated through metagenomics. After that samples were cultured on MRS media. For biochemical characterization gram staining, oxidase test, catalase test and Voges-Proskauer test were performed. In oxidase test kovacs reagent was used. In catalase and Voges-Proskauer test hydrogen peroxide and alpha naphthol were used respectively. To check the anti-diabetic drug sensitivity disc diffusion susceptibility method was used. The suspension of most commonly available drugs was used that includes metformin, acarbose, sitagliptin and combination of both sitagliptin and metformin. Three concentrations of each drug was used. The *Lactibacillus* species were isolated from diabetic and non-diabetic individuals. Metagenomic result indicated that there is a significance difference in gut microbiota of diabetic and non- diabetic individual. *Proteobacteria* were present in low quantities 3% in diabetic sample while 17% in normal sample. In taxonomic level of class *Protobacteria* was 15%, while in order *Lactobacillales* were 1%. At genus level the genus *Provetella* 9 was 30%. The *Lactobacillus* species in non-diabetic samples were *Protobacteria* 2% and *Lactobacillales* were 0.75%. While at genus level the genus *provetella* 9 is 27%. The most prevalent classes

were *Bacteroidetes* 40% and *Firmicutes* 20% in control sample. High expression of *Lactobacillales* was observed in heat map. For further conformation selective media, MRS media was used to facilitate the growth of *Lactobacillius* in collected fecal samples more ever gram staining protocol confirms the presences of gram positive bacteria. Oxidase, catalase and Voges-Proskauer test were performed which shows negative results. Anti-daibetic drug resistance was performed in order to check whether anti-diabetic drugs (Metformin, Acarbose, Sitagliptin and metformin+Sitagliptin) affect the growth of *Lactobacillus*. No zone of inhibition was observed against any of these anti-diabetic drugs tests. Based on the results it can be suggested that the variation in microbiome can be associated with the metabolic disorders such as diabetes.

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Abbreviations

α- GI: Alpha Glucosidase Inhibitors

FBG: Fasting Blood Glucose

GLP-1: Glucagon Like Peptide -1

GLP-1 RA: Glucagon Like Peptide -1 Receptor Agonists

HbA1c: Glycated hemoglobin

HDL-C: High density of Lipoprotein cholesterol

HOMA-IR: Homeostasis model assessment-estimated insulin resistance

IC: Insulin concentration

IL-1: Interleukin- 1

IL-6: Interleukin- 6

IR: Insulin resistant

ISI: Insulin sensitivity index

MRS: deMan, Rogosa and Sharpe (Selective media for growth of *Lactobacilli*)

SCFA: Single Chain Fatty Acids

SNP : Single Nucleotide Polymorphism

Sr1: serial number 1 (control sample)

Sr2: serial number 2 (diabetic patient)

TNF- α: Tumor Necrosis Factor Alpha

T1DM : Type 1 Diabetes Mellitus

T2DM : Type 2 Diabetes Mellitus

Chapter 1

Introduction

Metabolic diseases including obesity and diabetes has become a social problem for almost all countries [1]. Almost 62% increased risk of cardiovascular diseases had been evaluated due to advances in prevalence of diabetes in most recent decades [2]. Diabetes is the most prevalent and major public health issue. In 2017, it was assessed that 451 million individuals have diabetes around the world. The predominance of diabetes is expected to increment to 693 million by 2045 [3]. There had been a worldwide account of 387 million individuals those were effected by diabetes in 2014. It had been estimated in 2014 that 387 million individuals have diabetes around the world in which between 5% and 10% accounts for the Type 1 diabetes mellitus (T1DM). While Type 2 diabetes mellitus (T2DM) is more widespread and common type of diabetes. The rate by which diabetes mellitus specifically Type 2 diabetes is spreading reachedto 4–5% in Europe, 8–10% in America and more in the South Asia [4].

It has been estimated that diabetes prevalence is increasing worldwide, especially in the developing countries [5]. As Pakistan is developing country and it is facing increase growth in prevalence of the diabetes [6]. Diabetes is broadly categorized into two most common types that are Type 1 Diabetes which is dependent on insulin and other is Type 2 Diabetes which is insulin independent. There are certain types of diabetes which are less common that includes gestational diabetes, cystic fibrosis related diabetes and monogenic diabetes. Among these types of

diabetes, the T1D and T2D are two most talked about conditions and have somewhat various etiologies and pathogenesis [7]. The Type 1 diabetes is triggered by the damage of beta cells which resultantly secretes insulin by the T lymphocytes [8]. It is metabolic illness that is caused by the damaged islet beta cells which caused an abnormal secretion of insulin. This damage results in insufficiency of insulin that leads to the life-threatening hyperglycemia [9]. Type 2 Diabetes has an increased predisposition in South Asians. The danger of onset of diabetes is multiple times higher in UK for immigrant from Pakistan and Bangladesh and multiple times higher for Indian immigrants, with a related expanded danger of increased mortality rate and complexities as compared to the nativeresidents.The adverse effects of type 2 diabetes include chronic illness like, elevated blood glucose level, eventually damage to nerves and blood vessels that is because of impairment of insulin production [10]. The insulin, glucagon and digestive enzymes are secreted by healthy pancreas, and insulin regulates the glucose in body. In Type 1 diabetes insulin is not produced by the pancreas, and this can occur at any age. In Type 2 diabetes insulin is secreted by pancreas but body cells are unable to respond to insulin.

The carbohydrates are converted into blood glucose and insulin is needed to obtain blood glucose from blood which is major source of energy. The hemoglobin A1C (blood test to measure average sugar level over past 3 months) level of person tells the blood glucose level. Those people whose levels are among 5.7% or under 6.5% are pre-diabetic. The individuals whose level is about 6.5% or higher are diabetic. To reduce the blood glucose level, the insulin and other oral medications are given. In Type 1 diabetic patients are given insulin because their body cannot synthesize it. The insulin pumps are given to some individuals in small doses at specific intervals. The Type 2 diabetic patients take oral medication or insulin to maintain blood glucose level. In case of Type 2 diabetes the exercise and diet are very important to manage glucose level in blood and proper intake of medication [11].

Mostly the diabetes occurs in individuals having endocrine diseases and their body, secrete excess number of hormones such as glucagon, growth hormone, epinephrine

and glucocorticoids. Some of the hormones used as drugs like glucocorticoids, whichis used in immune system suppression and also in the chemotherapy [12].

Despite the fact the association and linkage studies identified the genes which are linked with the T2D but all genes are not associated with diabetes. Through Genome wide association studies in the various populations 70 loci has been identified to be linked with the Type 2 diabetes and gives positive linkage of Single Nucleotide Polymorphism (SNPs) and various mutations which effects expression of related proteins and risk associated with Type 2 diabetes development [13].

The composition of human gut microbiota has proven to be associated with various metabolic syndromes majorly in Type 2 diabetes and obesity. These microbes also participate in onset of gradual inflammatory effects through certain mechanisms that are associated with the gut barrier dysfunction, which is responsible for metabolic disorders. The human gut microbiome composition and diversity is associated with pathology of obesity. The presences of decreased number of *Bateroidates* and increased number of *Firmicuteshas* been found in obese mice. That are considered to be the large phyla of human micro biotic composition. The overall reason of impacting microbiota to treat T2D is upheld by the thought that the gut microbiota composition changes in diabetic patients [14].

It has been found that there are differences at phyla level that ratios of *Firmicutesto* the *Clostridium cocoides- Eubacterium rectale and Bacteroides, Prevotella* group are positively correlated with the plasma glucose concentration. There is also an increased prevalence of *Proteobacteria* and *Bacteroidetes* phyla in diabetic patients. The *Proteobacteria* contain pathobionts that may have role in inducing the low grade inflammation in the patients of diabetes [15].

In order to attain healthy microbiota one of the approaches is to take beneficial bacteria directly in the form of probiotics, which are defined as the live microbes which are taken to maintain health. The members of *Lactobacillus* phyla which includes *Lactobacillus acidophilus, Lactobacillus gasseri, and Lactobacillus rhamnosus casei,* have anti-diabetic effects [15]. Many strains of *Lactobacillus plantarum* species utilization plays important role in improving the glycemic control through

carbohydrate utilizing genes in the diabetic patients. Whereas it has been found that some species contribute in glucose intolerance like *Bifidobacterium breve, Bifidobacteriumanimals and Bifidobacterium longum* [16].

It has been observed that probiotics can be a helpful alternative to deal with this persistent illness in people because of the overall contrary effects of antimicrobial on gut and also risk of serious infections by antibiotic resistance bacteria. Moreover, the stable microbiota plays significant role in preventing T2D. Most of treatments that are currently in use especially pharmaceutical agents have focused on agents that directly influence the signaling pathways and regulate the glucose, now with more understanding of the root causes of Type 2 diabetes suggests that the human gut microbiome targeting would be more appropriate approach for treating T2D [16].

1.1 Problem Statement

Human health is directly associated with the change in composition or metabolic activity of gut microbiota. It is theorized that human gut microbiota specifically *Lactobacillus* species play a vital role in onset of diabetes which is an important beginning and possible potential use of probiotics to prevent and overcome the severity of Type 2 Diabetes mellitus (T2DM). Study of association of gut microbiome with diabetes mellitus and impact of antibiotic drug on *Lactobacillus* species can provide better understanding of *Lactobacillus* species role as therapeutic agents.

1.2 Aim and Objectives

The central aim of this study is to explore the differences in *Lactobacillus* species found in diabetic and non-diabetic people.

The objectives of the studies are following:

1. To identify the variations in *Lactobacillus* species found in diabetic and non-diabetic people

2. To characterize the *Lactobacillus* species by using biochemical tests

3. To analyze the effect of anti-diabetic drugs on most common *Lactobacillus* species isolated from diabetic and non-diabetic people

Chapter 2

Review of Literature

2.1 Type 2 Diabetes Mellitus

There are about 415 million individuals worldwide which has been experiencing diabetes with the exception of 193 million individuals have undiagnosed diabetes. Among all types of diabetes, T2DM shows the more spread rate of almost 90% and prompts micro-vascular and macro-vascular complexities that cause significant mental and actual trouble to the patients and careers and put a tremendous burden on health care systems. Although the knowledge of T2DM is well recognized but still its prevention, risk factors and predominance of its infection is kept on rising around the world. Through early detection the mortality and morbidity reduce by delaying or preventing diabetic complications [17].

The T2DM occurs when production of glucose in liver and impaired secretion of insulin occur. Physiological functions like autonomous and central nervous systems become altered which as a results secrete incretions and glucagon hormones in reduced amount. The presence of low grade inflammatory components in tissues like muscles, liver and adipose tissues are some common features of T2DM and obesity [17].

Metabolic inflammation results in production of cytokines like Interleukin 1 (IL-1), Interleukin 6 (IL-6) and Tumor Necrosis Factor alpha (TNF- α) by impairing

cellular insulin signals and results in diabetes and insulin resistance. Increase in weight also results in low-grade inflammation [17, 18].

2.2 Human Gut Microbiome

The human gastrointestinal tract contains 1,000 bacterial types and above 1014 microbial cells which are present in colon. At time of birth Gastro-Intestinal tract is sterile and during delivery process from vaginal and maternal fecal flora, bacteria begin to colonize. Initially the two common species of bacteria like *Streptococcus* species and *Escherichia coli* which are facultative anaerobes starts to colonize. Anaerobic environment is being created by these bacteria by metabolizing oxygen in gut. The feeding profile of infants determines the subsequent colonizing bacteria. There are certain factors that affect the frequency of various colonizing species in infant gut include female genital tract, mode of delivery, obstetric techniques and the type of feeding. The final and complex microflora develops at weaning [17]. The human adult gut contains many gram- positive cocci and non-sporting anaerobic species of bacteria and most predominant of which are the Bifidobacterium species, Bacteroides species, Fusobacterium species, Lactobacillus species, Eubacterium species and Clostridium species. The bacterial species which are present in comparatively less number includes Entrobacteriaceae, Enterococcus, dissimilatory sulphate reducing bacteria and methanogens [18].

2.3 Role of Gut Microbiota in Metabolism

The mutualistic relationship of host and gut microbiota is regularized by the complex network of interaction between metabolic, immune and neuroendocrine crosstalk. This complex crosstalk is stabilized by metabolites produced by microbial synthesis (described Table 1). Which act as signaling molecules to assists the host to perform various functions, such as gut microbiota facilitate the host by boosting its metabolic efficiency and provide it with more energy. To investigate

the potential of gut microbiota, 16S ribosomal RNA analysis and metagenomic sequences techniques are used on human fecal samples that showed the significant enhancement in metabolic rate of marco-nutritent and micro-nutritents [19].

TABLE 2.1: Metabolites contributed by gut microbiota and their respective functions.

Metabolites	Functions	References
Vitamins: Thiamine-B1, Riboflavin-B2, Niacin-B3, Pyridoxine-B6	Production of energy, formation of RBCs red blood cell, act as enzymatic cofactor in different biochemical reactions.	[22]
Polyamines: E.g, spermidine, spermine and putrescine,	Immune system functioning is enhanced. Polyamines: Maintenance of high rate of proliferation of Intestinal epithelial cells.	[23]
Derivativesof Indole E.g., indoxyl sulfate, indole-3-propionic acid (IPA) and Indole,	IPA acts as powerful antioxidant in the body, amyloid-beta fibril is inhibited by IPA, and shows cytoprotective effects against number of different oxidotoxins, also exhibits neuroprotective. Immune system functioning is enhanced	[24], [25], [26]

TABLE 2.1: Metabolites contributed by gut microbiota and their respective functions.

Metabolites	Functions	References
Acid metabolites of bile : E.g., lithocholic acid (LCA) and Deoxycholic acid (DCA),	Activate signaling pathways of the cell: bile acid metabolism, cholesterol, lipid, glucose, and energy	[27], [28]

The human gut microbiota is also involved in metabolism of various substances. Gut microbiota accomplish an important function in fermentation of soluble dietary fiber and unabsorbed starch. As a result of the fermentation process the end yield is short chain fatty acids (SCFAs),that provides 10% additional energy to host for effective metabolic process. About 70% of ATP production in colon is contributed by SCFAs [20].

Furthermore, microbiota of gut also facilitates by synthesizing micro-nutritents like vitamins that aids in metabolism of microbe and host. *Enterobactor lentum*, *Serratia marcescens* and *Entrococcus agglomeransare* gut bacteria that anaerobically produce vitamin K 2 (menaquinone). That is essential to perform various function like, lowering of cholesterol level, reduse the risk of cardiovascular diseases and decrease vascular calcification(As mentionedin table 2.1). Gut microbiota also play vital role in co-metabolism of bile acid within the host. These bile acids have antimicrobial properties which help in maintaining the gut micribiota and protecting the host from various types of infectious intities. The human adult gut contains many gram- positive cocci and non-sporting anaerobic species of bacteria and most predominant of which are the *Bifidobacterium* species, *Bacteroides* species, *Fusobacterium* species, *Lactobacillus* species, *Eubacterium* species and

Clostridium species. The bacterial species which are present in comparatively less number includes *Entrobacteriaceae, Enterococcus,* dissimilatory sulphate reducing bacteria and methanogens.

2.4 Diseaes Associated with Dysbiosis of Gut Microbiota

Dysbiosis is the alteration of regular or normal function of gut mirobiota under the influence of external environmental factors like dietary components, physical and psychological stresses and antibiotic consumption in coupled with some host factors. This dysbiosis leads to the enrichment of selective micobiota may be pathobionts that can alternate the regulation of microbial synthesized metabolites by causing wide range of diseases in host effecting specific cells, system or an organ (As described in table 2.2).

TABLE 2.2: Gut microbiome-linked human diseases and their particular dysbiotic features.

Categories of Diseaes	Specific Disease	Associated Dysbiotic Features	References
Autoimmune disease (immune mediated)	IBD Inflammatory-bowel disease - (IBS)	Virulent strains of gut microbiota are increased (species of Enterobacteriaceae	[33]
	Irritable bowel syndrome	and Bacteroides fragilis) and also increase	[34]
	Celiac disease	mucolytic strains like Ruminococcus sp.	[35]
	Diabetes Type-1	Actinobacteria species are decreased.	[36]

TABLE 2.2: Gut microbiome-linked human diseases and their particular dysbiotic features.

Categories of Diseaes	Specific Disease	Associated Dysbiotic Features	References
Metabolic disorders/ cardiovascular disorder	Obesity Type-2 diabetes Hypertension Atherosclerosis	Bacteroidetes show mixed results i.e. sometime increase or decrease or no effect. Lactobacillus sps increased	[37] [38] [39] [40]
Cancer	(CRC) Colorectal cancer	Enterotoxigenic strains of bacteroidesfragilis are increased, and fusobacterium and campylobacter sp which are pathobionts are also increase.	[41]
Neuropsy chiatric	(ASD) Autism spectrum disorder Parkinson's Diseases Depression	Increase of Bacteroidetes and Clostridium sp is observed, Lactobacillus and Desulfovibrio are also increased	[42] [43] [44]
Uremic disease	Chronic kidney disease	Increase of Proteobacteria strainsand Actinobacteria-strains also increase. Decrease of Lactobacillispecies.	[45]

TABLE 2.2: Gut microbiome-linked human diseases and their particular dysbiotic features.

Categories of Diseaes	Specific Disease	Associated Dysbiotic Features	References
Infectious disease	(CDI) Clostridium difficile infection	Increase of Clostridium difficelerstrains Decrease of general gut microbiota diversity	[46]

In normal gut microbiota one of the member of *Firmicutes*, named *Clostridium difficle* is a toxin and spoe producing gram positive anaerobe. By the catalytic activity of their toxins they damge the cytoskeleton alonge with integrity of colonic epithelial barrier causing the adverse effects, leadingto sudden inflammatory responses or death [29]. There are some microbiota species and their mechanisms of colonization resistance which confirms the protection of host against over growth of *Clostridium difficile* in normally gut microbiota. Some mechanisms are also being used for the reduction of number of these toxin producing bacteria that includes the bio-conversion of primary bile acids to secondary bile acids, which can help the host to increase its susceptibility against CDI [30].

The dybiosis in gut microbiota is also a major factor for the development of auto-immune disorders like celiac disease.T1DM is also categorized as autoimmune disorder caused by disturbance in gut associated mcribiome. Celiac disease is a chornic multifactorial immune mediated disorder of permanent intolerance of prolamine and dietary gluten in small intestine. Probiotic like *Latobacillius* and *Bifidobacterium* are helpful in reducing toxic effects and improving disease symptoms and providing encouraging results to overcome celiac disease [31]. Obesity is a global health hazard which is associated with higher energy consumption and lower energy expenditure leading to the accumulation of excussive fat with more

body mass index (BMI$\geq$ 30kg/m2). Obese person got higher risk of got effected by obesity related disorders like, low grade inflammation, T2DM,liver abnormalities and cardiovascular diseses. To overcome the severity of the disease the use of probiotic and prebiotic could be a promising therapeutic approach which is in need to be validated by clinical trial [32].

2.5 Gut Microbiota and their Role in Human Health and Disease

Human gut microbiota has been identified as the human health modulator to that extent that they are considered as an essential organ of human [47]. Emerging evidences proves that the human gut microbiome is crucial in physiological homeostasis maintenance. In microbiota of gut any alteration in composition and diversity of bacteria and ratio of *Firmicutes/Bacteroidetes* found to have a link with onset of hypertension [48].

There are various studies which confirms that human gut microbiota composition is associated with the atherosclerosis and also with arterial stiffness markers because of bioactive metabolites that are derived from the microflora. It has been found that phenyl-derived metabolites and indole is originated from gut microorganisms, it was found that metabolites that are derived from microflora are associated with the human postoperative cardiac complications and atherosclerosis [49].

The gut microbiota plays role in the T1D because they play significant role in the regulation of immune response. The human gut microflora composition depends on nutrients availability so the generation of the metabolites depend on intake of food. In Eα16/NOD mice it was found that they protect their offspring from the diabetes which is mediated by gut microbiota, this suggests that protection given by MHC/HLA alleles from autoimmune disorders may depends on gut microbiota.

In pre-diabetic persons the altered gut microbiota composition is categorized by reduced occurrence of *Clostridium genus* and *A. muciniphila*. It has been found

that the abundance of *A. muciniphila* is inversely related to risk of developing of Type 1 diabetes-related autoantibodies. The assembly of mucus was improved and the expansion of diabetes was delayed, when *A. muciniphila* was transferred to highincidence NOD colony. Therefore, the *A. muciniphila* can be proves a potential probiotic in Type 1 diabetes treatment [50].

2.6 Type 2 Diabetes Mellitus (T2DM) and Gut Microbiota

Prevalence of obesity with its related issues, for example, T2DM, has enhanced significantly throughout the world in advent of recent many years. Recent studies suggest that many metabolic disorders are directly associated with the altered gut microbiota composition and diversity. Large intestine has considered as home for the gut microflora that possess about 1011-12 bacterial concentrations for each gram of substance [51].

The human gut microbiome plays many physiological roles including synthesis of vitamins, extraction of nutrients, digestion, immune-modulation, metabolism and prevention against colonization by pathogens. The composition of human gut microbiome has been linked with the several metabolic syndromes especially with obesity and T2DM. Gut microbiota contributes to beginning of inflammation through certain mechanisms that are linked with the gut barrier dysfunction, which is responsible for metabolic disorders [52].

Among consistently studies the genera of *Bacteroides, Bifidobacterium, Akkermansia, Roseburiaand Faecalibacterium* were destructively accompanying with the Type 2 diabetes, while genera of *Blautia, Ruminococcus, and Fusobacterium* were positively associated with the Type 2 diabetes. *Lactobacillus* genus shows the maximum consistent results among studies.

Negative association between certain species like *B. pseudocatenulatum and B. longum,* and disease has been in patients who are cured with metformin [53].

2.7   Comparison of GutMicrobiota of T2DM Patients and Healthy Individuals

Individual with Type 2 diabetes lower number of *F. prausnitzii* was found than the healthy individuals. The *Bacteroides fragilis* and *Bifidobacterium longum* have shown no significant difference in their abundance. In comparison to healthy individual, patient with T2DMhave greater number of *Bifidobacterium* and lesser number of *Lactobscillius*. The alteration of intestinal microbiota and their link with T2DM has been found by dysbiosis in dominant fecal bacterial genera in T2D [54].

2.8   Bile Acid Metabolism and Association Between Gut Microbiota of T2DM

Chenodeoxycholic acid and cholic acid are two primary bile acids made from cholesterol in human liver. The microbiota present in human gut convert these main bile acids to tributary bile acids [55]. In large intestine the deoxycholic acid converts from the cholic acid due to *Clostridium* species. Metabolism of glucose is mainly done by these bile acids. These bile acids are activaters of many receptors like Nuclear farnesoid X receptor (FXR) and G protein coupled receptors. With the help of FXR the bile acid suppresses the expression of the gluconeogenic phosphoenolpyruvate carboxykinase [56]. Through study it has been observed that in diet-induced weight gain mice, when FXR gene was suppressed hyperglycemia and glucose tolerance were observed. These are also helpful in improving sensitivity of insulin and glucose clearance in adipose tissues. After the vertical sleeve gastrectomy, FXR play an important role maintenance of weight loss and also in improvement of glucose tolerance by shifts in the gut microflora composition and increasing the bile acid concentrations [57]. The sequestrates of bile acid prevents the gut reabsorption and hence results in LDL cholesterol reduction. By changing composition of gut microbiota of T2DM these compounds improves the control of

glycemic, and this results in improvements of increased secretion of the incretin hormones and hepatic glucose metabolism [58].

2.9   Role of Bacteria in T2DM

Twenty four studies have been conducted on T2D and bacterial microbiome which focused on the relationship between certain taxa and its corresponding disease. One finding which was most common was that certain bacterial genera such as *Faecalibacterium, Roseburia, Bifidobacterium* and *Akkermansia* were negatively associated with T2D. However, *Blautia, Ruminococcus* and *Fusobacterium* had a positive association with T2D. The most inconsistent results were regarding the detection of *Lactobacillus* genus. The studies also revealed that the ratio of *Bacteroidetes/Firmicutes* was previously suggested to be used as marker for T2D.

The association of Bifidobacterium and T2D was reported as a positive association in only one study other reported negatively. A negative relationship was found between patients after undergoing gastric bypass surgery or disease being treated with metformin and specific bacterial species like *Bifidobacterium dentium, Bifiobacterium pseudocatenulatum, Bifidobacterium bifidum* and *Bifidobacterium* .Various studies conducted on animal models have proven that Bifidobacterium has the ability to enhance glucose tolerance, however, it is yet to be used alone as probiotics for T2D [59]. *Bacteroides* was the second most frequently reported bacterial genus. To check the association of this genus with T2D as reported by eight studies out of which five cross-sectional studiesreported negative associations with the disease and the remaining three studies reported the opposite [59]. This opposition could be justified by the previously reported metformin's antibiotic effect and/or possible feedback mechanisms on gut microbiota as a consequence of enhanced human physiology.

However, in one study, 21 out of 23 OTUs of Bacteroides identified were shown to have a negative association with T2D. The species analysis of *Bacteriodes intestinalis* and *Bacteroides vulgatus* were lowered in number T2D patients. However,

it was seen that Bacteroides stercoris were increased in abundance after sleeve gastrectomy (SG) surgery in T2D patients with diabetes remission [60].

During various studies it has been reported that *Bacteroides uniformis* and *Bacteriodes acidifaciens* play a useful role in human glucose metabolism by improving glucose intolerance and insulin resistance. On the other hand, amongst all the studies *Roseburia, Akkermansia* and *Faecalibacterium* we're not proven to be as beneficial as the above groups.In further studies and investigations, *Roseburia* applied on species level also reported lower frequencies was found in T2D group than healthier groups and negative impacts with *Roseburia inulinivorans* disease have been observed. Amongst all others, just one study has reported a different result for *Roseburia intestinalis* [60]. Lower frequencies have been reported in the *Faecalibacterium* disease groups, however after several and different types of anti-diabetic treatments involving metformin and herbal medicine to bariatric surgery. In five studies conducted, four stated that on a species level of this genus assessment it was detected *F. prausnitzii* which also is a popular probiotic for colitis was negatively associated T2Dand there were very rare endeavors to use *F. prausnitzii* as a possible probiotic to treat metabolic diseases [60]. In one study conducted on mice specimens, *F. prausnitzii* administration to treat diet-induced metabolic disease without affecting blood glucose resulted in decreased liver fat inflammation and 12 improvement in hepatic function while the remission of diabetes after bariatric surgery species was linked with *Faecalibacterium* [61]. A newly discovered member of commensal microbiota is *Akkermansia muciniphila* which also showed a favorable effect on the glucose metabolism of the host [62]. In half of the T2D microbiome studies, an abundance of reduction in at least one of these five (*Akkermansia, Bifidobacterium, Roseburia, Faecalibacterium* and *Bacteroides*) phylogenetically distant genera in patients found and further microbiome studies suggested favoring majority of these bacteria to be used as probiotics for metabolic diseases in mice and their potential role beyond serving only as a biomarker, however it is rarely used in human beings [63].

From eleven out of forty-two studies, positive interrelations or escalation in disease of microbiota with T2D or hyperglycemia have been evident on the bases the

researchs can be held.

A positive association between T2D and *Ruminococcus, Blautia* and *Fusobacterium* have been shown. One study justifies this theory which found out that. *Ruminococcus* species SR1/5 was enriched by metformin treatment after bariatric surgery and diabetes remission and *Ruminococcus brommi* increased in abundance, whereas *Ruminococcus torques* decreased as concluded by another study. Another possibility reveals the major reasons for inconsistent results from different types of treatments between these studies [64]. In three out of four T2D case studies, reduction in genus of Blautia has been observed after bariatric surgery while it increased in individuals with disease. *Bacteroides, Bifidobacterium* are examples of various taxa at genus level, phylogenetically and higher levels (e.g., *Actinobacteria*) have been studied to show immunity from T2D at genus level while Lactobacilli shows only species- or strain-specific effects [65].

2.9.1 Gut Microbiota and Metabolism in T2D Patients

Inflammation caused by microbiota which affects energy homeostasis, lipid metabolism, gut permeability, glucose metabolism, as well as insulin sensitivity in the hosts by interacting with dietary components [65].

2.9.1.1 Modulation of Inflammation

Increased levels of chemokines, pro-inflammatory inflammatory proteins and cytokines are associated with T2D. Microbial products are increased by some gut microbes. A few examples include enhancement of low-grade inflammation by lipopolysaccharides (LPS) and metabolic endotoxemia. Some gut microbes also stimulate chemokines and anti-inflammatory cytokines. Bacterial Species of *Lactobacillus plantarum, Akkermansia muciniphila, L. casei, Roseburia intestinalis* and *Bacteroides fragilis* can improve the metabolism of glucose as this cytokine protects the muscles from insulin resistance due to aging. Induction of TGF-β can prevent the development of intestinal inflammation and heart burning [66].

Similarly, the bacterium *Bacteroides thetaiotaomicron* stimulates the expression of gene that code for T regulatory cell. R. intestinals has the ability to enhance production IL-2 which has the ability to restore diabetes and insulin sensitivity as well as promote the differentiation of T regulator cells, supress intestinal inflammation and induce TGF-B. *Bacteroides thetaiotaomicron* can also increase T regulatory cell gene expression [67].

To prevent inflammation another path is used by another microbes which are inhibition of chemokines and pro-inflammatory cytokines. Various other Lactobacillus species which include *L. plantarum, L. casei, L. paracasei* can decrease IL-1β, Monocyte Chemo attractant Protein-1, and Intercellular adhesion molecule-1, IL-8, CD36 and C– reactive protein. *L. paracasei* and *B. fragilis* hinder expression of IL-6. Accordingly, *Lactobacillus, Akkermansia*, and *Bacteroides* have been observed to suppress TNF-α. The activity of NFkB is inhibited by *F. prausnitzii*and by *L. paracasei*. Likewise, butyrate like *Roseburia* and *Faecalibacterium* produce bacteria and also inhibit the activity of NFkB. *Roseburia intestinalis* and *Lactobacillus casei* decreases another pro-inflammatory cytokine IFN-σ, while *Roseburia intestinalis* can constrain IL-17 production. In the monoassociated mice the Th1, Th2 and Th17 cytokines are decreased by *Bacteroidesthetaiotaomicron*. Furthermore, the detrimental microbes in T2D (pathobionts), like bacteria *Fusobacterium nucleatum* and *Ruminococcusgnavus* in T2D pathobionts can potentially increases the inflammatory cytokines [67].

2.9.1.2 Gut Permeability

Translocation of gut microbiota products in to blood stream is caused by metabolic endotoxemia in T2D patients, which is a result of increased intestinal permeability [68].

Studies have shown that two bacterial species *B. dorei* and *Bacteroides vulgatus* can reduce amelioration and production of endotoxemia, reduce LPS and gut permeability in mice models as these species up regulate the communication of tight junction genes in the colon [69]. Furthermore, *Akkermansia muciniphila* which

is another probiotic bacterium that decreases gut permeability but by using a different mechanism than the above-mentioned bacterial species.

It uses extracellular vesicles through AMPK activation in the epithelium to improve intestinal tight junctions. The outer membrane protein (Amuc-1100) improves gut integrity, enhances the expression of tight junction protei-1 (CB1) and occludin .The outer membrane protein (Amuc-1100) also reduces systemic LPS levels and gut permeability by inhibiting the cannabinoid receptor type 1 (CB1) [57].

Even though a particular bacterial component was not originated for the bacterium *Faecalibacterium prausnitzii*, it was found that the supernatant from cultured *Faecalibacterium prausnitzii* improves intestinal barrier functions by enhancing the tight junction protein expression in colitis model [56]. Finally, butyrate being produced by the bacterial species *Faecalibacterium* and *Roseburiaalso* have the capability to decrease gut absorbency by use of PPAR-σ pathways and serotonin transporters [57].

2.9.1.3   Glucose Metabolism

Gut microbiota alters the insulin resistance and glucose homeostasis in metabolic organs such has muscle, fay and liver directly affecting T2D. One of the bacteria found in the gut, *Bifidobacterium lactis* when used as a probiotic can not only decline the communication of hepatic gluconeogenesis related genes but also increase synthesis of glycogen [58]. *B. lactis* can also improve uptake of insulin-stimulated glucose and translocation of glucose transporter-4 (GLUT4).

Additionally, one other bacteria of the gut, *Lactobacillus gasseri* BNR17 has a possible antidiabetic effect as it increases expression of GLUT-4. Hepatic flavin monooxygenase 3 (Fmo3) is an enzyme 15 of xenobiotic metabolism and its knockdown has been studied in insulin resistant mice to show that it can prevent the development of hyperlipidemia and hyperglycemia [70]. *Lactobacillus plantarum* and *Akkermansia muciniphila* have been showed to reduce the expression of Fmo3.

Lactobacillus casei has multiple functions such as reduction of hyperglycemia using the up regulation of GlyRa1, C1c1-7, GABAAa1, SLC26A6, C1C1- 7, CFTR and Bestrophin-3 through a bile acid-chloride exchange. By increasing the mRNA level of AMPK, Akt2, insulin receptor substrate 2 (IRS2) and phosphatidylinositol-3kinase (PI3K) it can help in improving insulin resistance [53].

It also plays a vital function in the white adipose tissue as it decreases the growth factor binding proteins-3 (IGFBP-3) and caco-2 cells which are insulin-degrading enzyme in insulin [72]. One *Lactobacillus* species and *L. rhamnosus* improves the adiponectin level in the epididymal fat which in return improves Insulin sensitization [48]. Potent alpha-glucosidase inhibitory activity possessed by *Akkermansia muciniphila* and *Lactobacilli* species reduces postprandial hyperglycemia by preventing the breakdown of complex carbohydrates [73].

Bile salt hydrolases are produced by *Lactobacillus* and *Bifidobacterium* which metamorphose conjugated bile salts in to de-conjugated bile acids that further prompt the production of GLP-1 by converting de-conjugated bile acids in to secondary bile acids [74]. Butyrate when acting as a ligand for GPCR41 and GPCR43 which are G-protein coupled receptors encourage release of gut hormones PYY, GLP-2 and GLP-1. Therefore, it can be concluded that the gut microbiome and its products have the ability to improve glucose tolerance and insulin resistance and modulate enzymes and gut hormones [75].

2.9.1.4 Synthesis, Oxidation and Energy Expenditure of Fatty Acids

Reduction of fatty acid production and increase *theiroxidation* and energy expenditure ameliorates T2D and obesity. Microbes such as *L. gasseri, A. muciniphila* and *B. acidifaciens. A. muciniphila* increase the fatty acid oxidation in the adipose tissue by increasing level of 2-oleoyl-glycerol, 2- palmitoylglycerol, 2-acylglycerol [76]. Using TGR5-PPAR-α pathway, *Bacteroides acidifaciens* also accelerates oxidation of fatty 16 acids in the adipose tissue. In this sense. *Lactobacillus gasseri* increases fatty acid oxidation genes and reduces the genes related to fatty acid reduction, hence, reduces obesity [77]. In diabetic rodents, *A. muciniphila* and *L.*

casei, reduces serum level of *maonoidialdehyde* (lipid marker for oxidative damage). Butyrate not only inhibits the histone de-acetylation process in muscles, promoting fatty acid oxidation but it also works with two other SCFAs in the liver and adipose tissue, acetate in addition to propionate, decrease PPAR-σ expression increasing fatty acid oxidation. Therefore, with the above knowledge it can be seen that bacteria from the gut microbiome have a positive effect on T2D modulate energy expenditure and fatty acid metabolism [78].

2.9.1.5 Bacteria and their Combined Effects

Some bacteria can alter the physiology by cross feeding or by increasing the number of other potential beneficial bacteria. In rats, *L. rhamnosus* increases the abundance of *Bifidobacteria* in the cecum which then has cross feeding interactions with bacterial species like *Roseburia* and *Faecalibacterium*. Butyrate producing bacteria can also be increased in number by *L. casei* [79].

2.10 Diabetes Mellitus Drug Therapy and Gut Microbiota

Obesity associated diabetes along with energy-rich diets increase the ratio of Firmicutes to Bacteroidetes in the intestines. Obesity also results in the decrease of microbial diversity. Dysbiosis is induced by T2D which results in the drop of number of butyrate producing bacteria and *Akkermansia muciniphila* [80].

2.10.1 Biguanides

Metformin is widely used due to its insulin sensitizing effects and lowering glucose properties. It is believed that it regulates uptake of glucose and the glycolysis and synthesis of glycogen in liver. Metformin increases the Glucagon Like Peptide -1 plasma levels and also enhances GLP-1 expression in the pancreas islets.

Metformin has positive effects on short chain fatty acids (SCFA) producing bacteria which include *Bacteroides*, 17 *Butyricoccus* as well as other bacteria such as *Allobaculum* and *Lactobacillus* [81].

2.10.2 Alpha-glucosidase inhibitors (α-GIs)

These drugs alter the nutrient source of bacteria by the reduction of postprandial hyperglycemia and in the small intestines delay the digestion of carbohydrates [82]. α-GI acarbose has the ability to block the growth of *Escherichia coli* on maltose. Acarbose reduces the processing of starch and its absorption which results in number of butyrate producing bacteria and starch fermenting bacteria. Therefore, concentration of butyrate and starch are increased in the fecal samples of patient staking this drug [83]. Acarbose elevate the bacteria number like *Bifidobacterium, Dialister, Lactobacillus* and SCFA- producing bacteria such as *Prevotella, Faecalibacterium* and decreases the abundance of *Bacteroidaceae, Enterobacteriaceae* and *lecithinase positive Clostridium* [84].

2.10.3 Incretins Based Drugs

Intestinal cells secretes incretins after each meal which play key role in regulation of glucose levels in the blood and it also reduces appetite. This is due to the pleiotropic metabolic activity they have. To date, only two incretins have been identified which are GLP and GLP-1, both of which are secreted immediately after meal absorption by the L and K cells of the intestines [85].

2.10.4 GLP-1 Receptor Agonists

GLP-1 RA are considered as an altered peptide which share the homology with GLP-1. Apart from the fact that GLP-1 RA that is inserted in the gut microbiota subcutaneously gives no direct effects on the structure of gut microbiota is considerable displacement of the bacterial structure in a mice was seen when treated

with liraglutide. The postulate suggested that the GLP-1 level can alter the internal environment of a gut lumen such as including the PH and ultimately the composition of gut microbiota. GLP-1 basically effects gut transit time as well as gastric emptying rate [86].

In a mice with high fat diet, it was seen that liraglutide tends to lower the microbial diversity in it, showing that the Firmicutes are enhanced whereas, the *actinobacteria, proteobacteria* and the *Bacteroidetes* phyla are deprived in a microbial diversity. However, it was also observed that in a high-fat fed or an induced diabetes mice, the treatment of mice with 18 liraglutide induces the improvement in a total of 13 phylotypes, which includes the genera of *Allobaculum, Turicibacter, Anaerostipes, Blautia, Lactobacillus, Butyricimonas, Desulfovibrio*, while a decrease in 20 phylotypes in the orders *Clostridialesand Bacteroidales* is seen that, In a like manner to GLP-1 RA, an induced weight loss is another effect of liraglutide. It was observed that liraglutide enriched only the *Lactobacillus, Turicibacter, Blautia* and *Coprococcus*, whereas the phylotypes related to obesity were depleted such as *Parabacteroides Erysipelotrichaceae incertae sedis, Marvinbryantia, Roseburia* and *Candidatus Arthromitus* [87].

2.10.5 DPP-4 Inhibitors

Sitagliptin is one of the DPP-4i that helps to reinstate gut microbiota at the level of phylotypes in the rats that are induced with diabetes but have no effect on their body weight. Sitagliptin is an inhibitor that induces the relative abundance of *proteobacteria* as well as *bacteriodetes* while depletes the abundance of firmicutes. However, at the genus level, it is observed that sitagliptin effects the Short Chain Fatty Acid producing bacteria. The diabetic rat stools treated with sitagliptin were seen to be improved in *Roseburia*, however, showed deprived Blautia whereas, the number of *Clostridium* did not change. It was observed that the relative abundance of *Lactobacillus* and *Bifidobacterium* as probiotics depleted in the stool of a diabetic mice [88]. However, it was observed that sitagliptin only prevented the *Bifidobacterium* to be reduced while it intensified the decrease of

Lactobacillus. Another type of DPP-4i known as saxagliptin, have opposite effects on the phylotypes of microbiota than sitagliptin. It is seen that the feces of high-fat fed mice or the ones with induced diabetes mice that are treated with saxaliptin are improved in Firmicutes, due to the genera *Turicibactor, Lactobacillus* and *Allobaculum.* They contain reduced amount of phylum *Bacteroidetes* due to depletion in *Bacteroides* and *Prevotella* [87].

2.11 T2D Therapy and Microbiota

Association between gut microbiota with drugs thought to be an interesting area of research. It is observed that the anti as well as non-antibiotic anti-diabetic drugs can modify the gut microbiota and ultimately improves the diabetes. In a like manner, the 19 microbiota can affect the pharmacodynamics of drugs and other chemicals, positively or negatively through several mechanisms [87, 89]. However, few studies have studied how changing the gut microbiota (through prebiotics and / or probiotics) alters the effects of diabetes medications. A recent study stated the possessions of a probiotic *Bifidobacterium animalis* species and lactis 420, prebiotic polydextrose and its association with sitagliptin in diabetic mice [90].

The fusion of sitagliptin with prebiotics as well as probiotics were successful in lowering the several type 2 diabetes parameters. A relevant study in zucker diabetic mice showed the combination of prebiotic polysaccharide with metformin and sitagliptin, known as anti-diabetic drugs decreased hyperglycemia as well as adiposity rather than using the drugs only [91].

In one of the other studies, a combination of metformin and prebiotic was given to treat a streptozotocin-induced mouse, that was diabetic. The results showed the enhancement in the blood glucose in fasting, insulin resistance as well as glucose tolerance, by using this combined therapy, rather than using metformin alone.

All the previous advancement and research showed a new way to figure out the association of gut microbiota and anti- diabetic drugs such as metformin, sitagliptin [91].

2.12 Probiotics and Type 2 Diabetes

2.12.1 Association between Intestinal Microbiota and Probiotics as well as their Effects on T2DM

A human body has tens of trillions of intestinal microbiotas that includes approximately 1000 various species. Intestinal microbiota is thought to be implicated in different diseases and health. The disease associated with them include metabolic imbalances, encompassing the glucose intolerance as well as insulin resistance, type 2 diabetes to metabolic disability and obesity [92].

Gut microbiota showed significant role in permeability of gastrointestinal mucosa and host immunity that are the vital factors in T2DM. The imbalances of intestinal microbiota known as dysbiosis can cause the development of adipose tissue, extraction of diet energy, as well as synthesis of fats, which can ultimately lead towards metabolic disabilities. These also contribute towards enhanced adiposity, oxidative stress as well as metabolic endotoxemia [93].

Currently, the modulation of intestinal microbiota seems like a compelling tool to prevent and treat dysbiosis that is mainly associated with obesity and other metabolic syndrome.The advantageous modulation of the intestinal mucosa can be gained by introducing probiotics, as well as prebiotics or the other way is by transplantation of fecal microbiota. The beneficial effects of probiotics as a dietary supplement as well as functional food on health has made it very popular nowadays [93].

In food industry live microorganism like *Lactococcus (L.)*, *Lactobacillus (Lb.)*, and *Bifidobacterium (B.)* are used as probiotics and thought to be inhabited in gut and hence, tends to show the enhancing health characteristics. The probiotics that are taken orally are integrated with the microbiota in the intestines in two ways, either permanently or transiently [94]. Despite of the modulation of microbial diversity in intestinal microbiota, the probiotics are also helpful in treatment of diseases such as T2D. The introduction of *Lactobacillus* species is linked with the lowering

of expression of genes related to pro inflammation, shown in Appendix table (5.1) [95] to [101].

2.12.2 Probiotics Administration for the Intervention of T2DM

The intervention of probiotic in T2D has seen to have beneficial effects through several mechanisms . Different studies shows that for treatment and prevention of metabolic disorder like T2DM probiotics have been used as dietary supplement [102]. Probiotics are thought to improve the integrity of gut, lipopolysaccharide levels are decreased, as well as enrichment of insulin sensitivity [103].

2.12.3 Enhancement of Function of Gut Barrier and Secretion of Incretins using Probiotics

One of the infectious traits of type 2 diabetes is the elevated permeability in gut, as well as lowered inflammation because microbiota of gut is an important factor in developing systematic inflammation. The result of dysbiosis caused by gut microbiota results in the increased gut permeability that results in hyperglycemia and inflammation caused by the bacterial endotoxins [104]. The occurrence of protein kinase C isoforms is increased by probiotic lactobacilli which enhances the integrity of epithelial barrier [105].

Incretins are termed as metabolic hormones which are responsible for the secretion of an insulin that ultimately decreases the blood glucose levels. The two incretins termed as Glucagon Like Peptide -1 and Glucose- dependent- insulinotropic- polypeptide. The probiotics are responsible for the secretion of peptide tyrosine as well as the glucagon-like peptide-1 through SCFA from the gut [89]. The intervention *Lactobacillus* probiotic to the rats exhibited the elevated Glucagon Like Peptide -1 levels and Glucagon Like Peptide -2 which resulted in intestinotrophic effect which was in result of the release of hormone that was gastoinstestinal [106].

2.12.4 Effects of Probiotics in Insulin Sensitivity and Energy Metabolism

Probiotics are termed as an important part of gut microbiota as they play an important role in energy gain and metabolism. A probiotic combination of *L. acidophilus* and *S. cerevisiae* results in the enhancement of expression of mRNA of CPT1 known as carnitine palmitoyl transferase-I, acetyl-CoA acetyltransferase II, CPT2 known as carnitine 25 palmitoyltransferase II, acyl-coenzyme A oxidase, however, it downregulates the expression of mRNA of fatty acid synthase, lipoprotein lipase that is mainly involved in the metabolism of lipids [107]. The bacteria *B. lactis* is involved in the enhancement of insulin-stimulated uptake of glucose as well as displacement of GLUT4 through the AKT and TNF as an insulin signaling pathways. However, *B.lactis* is involved in the elevation of the mRNA gene like GLUT4 and pp-1 which are present in the tissue of skeletal muscle, meanwhile it also lowers the levels of mRNA by genes like hepatic gluconeogenesis-regulated genes that are PCK1 and G6PC present in the liver [108].

Composition of bile acids are affected in probiotics that tends to bind with the cellular receptors and then in return it activates various different pathways that are mainly take part in energy metabolism of lipids and glucose homeostasis. Furthermore, probiotics are involved in elimination of host calories as well as they are involved in producing short-chain fatty acids that helps in regulation of intestines and also influences the energy mechanism of the host [108].

2.12.5 Modulation of Host immune Responses by Probiotics

Probiotic play significant role in boosting and conservation of the body's immune system. The bulk of immune cells resides in intestine. The previous studies give clear evidence that the hereditary and gained immune responses are regularized and boosted by the strains found in the probiotics. The most common probiotics found in food industries (*L. casei, L. johnsonii, L. rhamnosus, B. lactis, B.*

animalis, and *S. cerevisiae*) acquired immunity of the host [109]. Some of the strains help to regulate the gene expression which is linked to an anti-inflammatory behavior and degrade the pro-inflammatory gene which eventually affects the inflammatory signaling pathways [97]. From the past research, it can be concluded that the activity of *L. plantarum* Toll-like receptor stimulation is hinged on the culture state and associated with IL-10-inducing activity. The fetal immune response in infants is 26 greatly affected by the provision of the probiotic organism, some of them are breast milk immunoglobulin A (IgA), cord blood interferon (IFN)-c levels, and transforming growth factor (TGF)-b1 levels. The fight with chronic inflammation in infants is greatly influenced by consuming formula with B. lactis Bb12 augmented secretory IgA found in feces [110]. Probiotics stimulate macrophages, natural killer (NK) cells, antigen specific cytotoxic T lymphocytes by enhancing the nonspecific immune response. Probiotics and their metabolites can maintain pro-inflammatory and anti-inflammatory immune response by interacting with varieties of immune cells such as antigen-presenting cells and T-cell by conferring their immune-regulatory functions [111].

2.13 Relationship between *Lactobacillus* and T2D

Among the most potential probiotic bacteria lies the *Lactobacillus* that is diverse and also it contain the most numbers of OTUs in the gut of a human. A total of 6 studies showing the relationship of T2DM and *Lactobacillus* genus was studied by cross sectional studies. In which five shows their positive relation with *Lactobacillus* genus [112].

The administration of *Lactobacillus* specie and the associations of this genus was observed to be more specie specific, which showed that some of the species such as while *L. acidophilus,L. gasseri, L. salivarius* were enriched due to this genus, however the specie was deprived in patients of T2DM. Furthermore, various species from this specific genus were used to be tested as probiotics [113]. The bacterial specie known as *L. plantarum* widely occurs in fermented foods, and according

to several studies in the animal systems, it has been stated that L. plantarum is involved in enhancement of glucose metabolism both, the diet induced as well as genetic T2D mice [114]. Our GI tract contains trillions of gut friendly microbes that pose potential advantages to our body in terms of improved physiology and body's immunity [115]. Among the most dominant *Lactobacillus* species in the gut *L. johnsonii and L.gasseri* are involved in anaerobic oxidation in GI tract. *L. johnsonii* is a gram-positive bacillus shaped non spore forming bacteria that promote protein and complex carbohydrates digestion and produce a variety of fatty acids that contribute to 15% calorie intake by men per day 27 *L.gasseri* have been regarded as true autochthonous species of human intestine and follows a commensal intestinal relationship with host i.e., human intestine [116], [104], [117].

2.14 Research Gap

Many studies have monitored the gut microbiota and investigated its relationship with T2DM in different populations, inconsistent results describing microbial differences have been reported between diabetic and healthy individuals. Association of inconsistent results with anti-diabetic drugs have not been directly reported or evaluated yet.

2.15 Research Questions

1. Which type of microbial diversity exists in T2DM patients and healthy people?

2. Is there any association of - drugs with *Lactobacillus* population?

Chapter 3

Research Methodology
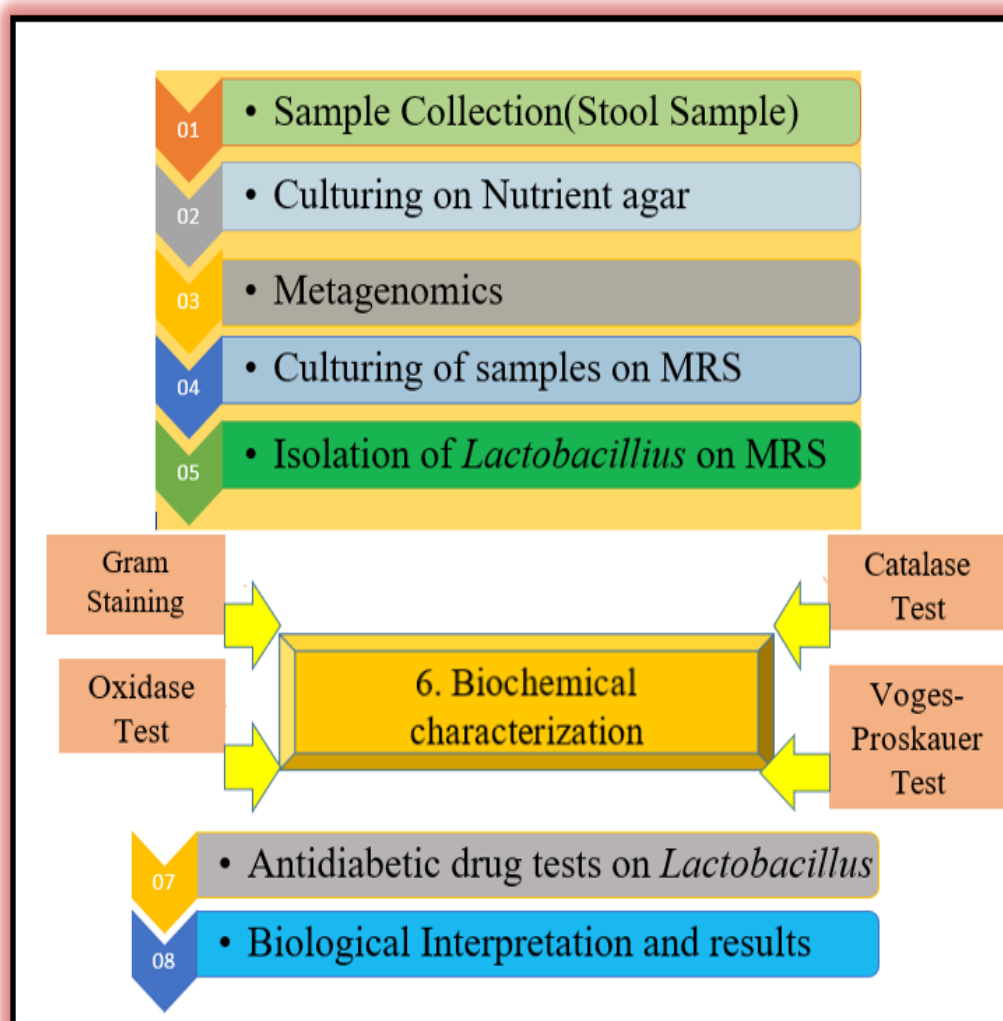
3.1 Materials

3.1.1 Chemicals

PBS buffer (Sigma), MRS media (Biolab), agarose (Bioworld), glycerol (Merck), crystal Violet (Sigma), kovac's reagent (AnalaR), hydrogen peroxide (Paradise pharma) Alphanaphthol (Sigma), MR-VP broth (Alpha biosciences), ethanol (AnalaR), Safranin solution (Sigma), Gram Iodine (Scharlau)

3.1.2 Apparatus

Petri plate, beaker, conical flask, wire loop, spirit lap, micropipette, Cotton swab, Measuring cylinder, spatula, microscopic slides, dropper, falcon tube, eppendorf and reagent bottles.

3.1.3 Equipment

Laminar flow hood, incubator, weighing balance, autoclave, vortex, microwave, water bath and pH meter.

3.2 Inclusion and Exclusion Criteria

3.2.1 Inclusion Criteria

All participants provided their signed written consent. Fifty T2D patients and fifty control subjects with below mentioned properties were included. Diabetic subjects and control subjects

- Broad age (20–86 years)

- Body mass index (BMI) (17–36 kg/m2)

- Patients will be defined as T2D if they have disease duration of at least 5 years duration but otherwise healthy.

3.2.2 Exclusion Criteria

Participants were excluded if there was any evidence of below mentioned disorders,

- Significant cardiovascular complications

- Significant renal, hepatic, or neurological disease

- Cancer, Pregnancy, Diarrhea [118].

3.2.3 Designing a Questionnaire

Questionnaire was designed based on above mentioned inclusion and exclusion criteria mentioned in appendix 6, [119].

3.3 Sample Collection

Hundred stool sample was obtained in a sterile container of size 50ml made of glass. Samples were immediately brought to the lab and were kept in freezer at -40 C.Stool specimens did not contain urine or water.

FIGURE 3.2: Sample Collection

3.4 Ethical Clearance

Ethical clearance was received from ethical committee of "Department of Bioinformatics and Biosciences".

3.5 Culturing on Nutrient Agar

A synthetic environment that helps to simulate natural growth conditions was necessary for bacterial growth on culture media. For extensive growth of bacteria nutritent agar was used. By using a measuring balance, 5.6g of nutrient agar was measured out and 200 ml of distilled water were added. The combination underwent a 15–20 minute autoclave at 121C. A homogeneous 20ml volume of autoclaved media was poured into sterilized petri dishes. Through the use of micropipette tips, petri dishes were filled. 5 ml of the prepared sample were poured into 10 Petri plates containing nutrient agar, and then the sample was placed thoroughly by using a spreader. To avoid moisture, plates were incubated in an upside-down position for 24 hours on 37 °C

3.6 16s rRNA Sequencing

Two samples, one of diabetic patient and other for healthy individual was sent for metagenomic analysis

3.7 Preparation of MRS Media

Fecal sample was taken using swab, entire tip of the swab was inserted into the stool sample. PBS (Phosphate Buffer Saline) was made by adding 23g of NaCl, 62.8g of Na_2HPO_4 and 69.6g KH_2PO_4 in 400ml of distilled water. After that, its PH was brought to 7.4 by adding HCL in it and PBS was transferred to

eppendorf tubes. Sample were transferred into eppendorf tubes and 2ml of PBS buffer was also added to each after which they were homogenized and then it was vortex mixed.2ml of PBS buffer was also added to each after which they were homogenized and then it was vortex mixed.

FIGURE 3.3: Preservation of Diabetic and non-diabetic samples in PBS (Phosphate Buffer Saline)

The isolation of *Lactobacillus* from fecal sample was done by using MRS medium, which is a selective culture medium designed to favor the growth of *Lactobacilli*. MRS agar of 70.25 g was added in 4000ml distilled water. Then the prepared media was autoclaved inside a sterile container at 121°C for two hours. After that media was poured into autoclaved Petri plates uniformly within the laminar flow and it was cooled for 10 minutes to solidify the liquid media.

3.8   Media Inoculation

Each bacterial sample was grown on MRS media to get colonies of bacteria associated with diabetic and non-diabetic individuals. By using a sterilized loop, each sample was transferred to a petri dish in laminar flow hood and was mentioned plate as diabetic and non-diabetic individuals. Then sample was stored for 24 hours at 37C within the incubator in upside down direction to avoid moisture. The inoculum was selected based on the colonial morphology.

3.9 Purification of Bacterial Strains

The *Lactobacillus* strains were selected for molecular and biochemical characterization. For this purpose, the MRS media in conical flask autoclaved for 2 hours at 121 C for sterilization. Then the media was taken into laminar flow and pouring the media into autoclaved petri plates, after solidification. Then the culture strains were taken and streak into the MRS agar.

3.10 Biochemical Characterization

For biochemical characterization different tests were performed that where describe below.

3.10.1 Gram Staining

3.10.1.1 Preparation of Crystal Violet Solution

20g of crystal violet were dissolved in 100ml ethanol to make crystal violet solution. The solution was preserved in falcon tubes for staining purposes.

3.10.1.2 Preparation of Gram Iodine Solution

32 0.3g of iodine pearl, 6.67g of potassium iodide and 1g of sodium bicarbonate were dissolved in 100 ml of distilled water. In this way, a gram iodine solution was prepared.

3.10.1.3 Gram Staining Procedure

The gram staining is used for differentiation of gram positive and gram-negative bacteria. A glass slide was taken and clean with 75 percent ethyl alcohol. Then

dilution was prepared by adding a loop of purified bacterial culture in 2ml of distilled water in the beaker. Then drop of bacterial suspension on the middle of the slide was added after labeling the slide. Then slide was allowed to dry. Then bacteria were heat fixed on the spirit lamp for 60 seconds. A drop of crystal violet was added on the heat fixed bacteria and left for 30 seconds, then it was rinsed with water. Then 3-4 drops of gram iodine were added to slide and left for one minute. After that slide was rinsed with distilled water. The slide was then washed with decolorizer, which contain 95% ethanol, it was run through the stained area so that it decolorizes the stain and no more color washes out, the slide will again be washed with water. Then 3-4 drops of safranin were added.

3.10.1.4   Preparation of Safranin Solution

2.5 grams of safranin was dissolved in 100 ml of 95% concentrated ethanol for the preparation of the stock solution the working of the solution was obtained by adding 1% by adding one part of the stock solution in all five parts of the distilled water.

3.10.1.5   Preparation of Distaining Solution

For distaining solution 5 ml of 95% ethanol and 50 ml of acetone were mixed. In this way, 100 ml of distaining solution was prepared.

3.11   Tests for Gram Positive Bacteria (*Lactobacillus Species*)

3.11.1   Oxidase Test

For carrying out oxidase test, Kovacs reagent was used. A piece of filter paper was soaked in the reagent and left to dry out. Using a sterilized wire loop, the isolated

bacterial colonies were transferred from the plates to the filter paper. After, 2 minutes the results were noted.

3.11.2   Catalase Test

A catalase test was used for *Lactobacillus* identification which is gram positive bacteria. For this test a drop of hydrogen peroxide solution was poured into a microbial slide. Sterile loop was used to take several colonies of 24 hours incubated samples and sink in hydrogen peroxide solution. The immediate bubbling showed the presence of gram-positive bacteria, whereas, no bubbling represented the presence of gram-negative bacteria. The process was repeated for all samples.

3.11.3   Voges-Proskauer Test

The samples that were inoculated for 24 hours prior were added in the test tube using a sterile loop. 6 drops of 5% alpha-naphthol were added and mixed well to aerate. 2 drops of 40% potassium hydroxide were then be added and mixed well to aerate. The test tube was shaken vigorously and the color change was observed.

3.12   Effect of Anti-Diabetic Drugs on *Lactobacillus* Species

The effect of anti-diabetic drugs on the *Lactobacillus* species was analyze using disk diffusion susceptibility method. The isolated *Lactobacillus* species were streaked on MRS media plates. The suspension of most common drugs available for use such as metformin (Biguanides), acarbose (Alpha-glucosidase inhibitors α-GIs), sitagliptin (DPP-4 Inhibitors) and metformin with sitagliptin were prepared, 3 concenrations were prepared for each anti-diabetic drug. The anti-diabetic disks for each drug were placed on the petri plates containing the *Lactobacillus* species using sterile forceps with in equivalent space from other disks.

TABLE 3.1: Commercially available anti-diabetic drugs and their recommended usage

Commerically Available Antidiabetic Drug	Recommended Dose Usage
Sitagliptin (Trevia)	25mg
	50mg
	100mg
Metformin (Glucophage)	25mg
	50mg
	100mg
Acarbose (Glucobay)	25mg
	50mg
	100mg
Sitagliptin and metaformin (Treviamet)	275mg
	550mg
	1100mg

The petri plates were protected at 35C for 24 hours. The zones of inhibition around each of the anti-diabetic disk were sedate to the nearest millimeter. The length of the zone is correlated to the susceptibility of the isolates and to the diffusion rate of the drug through the MRS media. The zone diameters of each drug will be inferred using the criteria published by the Clinical and Laboratory Standards Institute (CLSI). The results of the disk diffusion test were "qualitative" in that category of susceptibility in the form of susceptible, intermediate or resistant.

Chapter 4

Results and Discussions

4.1 Sample Collection

Sample collection was carried out during the month of January. After the ethical clearance, stool samples had been collected from city of Islamabad (random sampling). There were 100 samples in which 50 were diabetic and 50 were controlled. The samples were brought in a sterile container of size 50ml and immediately transferred to in 2ml of PBS buffer after that they were homogenized and then it was vortex mixed and kept in freezer at -40 °C. Then nutrient agar was used to facilitate the bacterial growth after that selective media MRS for the growth of Lactobcillus was also being used, followed by biochemical testing and metagenomic analysis. The results are now described in a proper order.

4.2 Culturing on Nutrient Agar

Bacteria were isolated from both health and diabetic people. A general purpose nutrient agar media was used to culture the isolated bacteria. This is used for the growth of many kinds of bacteria. The chemical composition of nutrient agar includes beef extract, peptone and agar.Its simple formulation aids in nutrient value that favors bacterial genome replication and the growth of gram-positive

and gram-negative bacteria. The results indicate the growth of variety of bacteria in diabetic and healthy individuals.

4.3   Metagenomics

To characterize the microbiome with and without diabetes, 16S rRNA gene sequencing was used to investigate 2 fecal samples from healthy and diabetic people. Sr 1 represent the control sample and Sr2 was the diabetic sample. The approximately 21,476 genome size was obtained. The GC contents were 54.4%. Number of coding sequences were 24. The number of RNAs was 3. The sequence similarity was 98% and these sequences were clustered in 10 genera and 9 phyla.

4.4   Multiple Sequence Alignment of Sequence

The multiple sequence alignment was done in ClastralW of Sr1 and Sr2 and the results of multiple sequence alignment showed that there was variation in sequence with name Sr2.
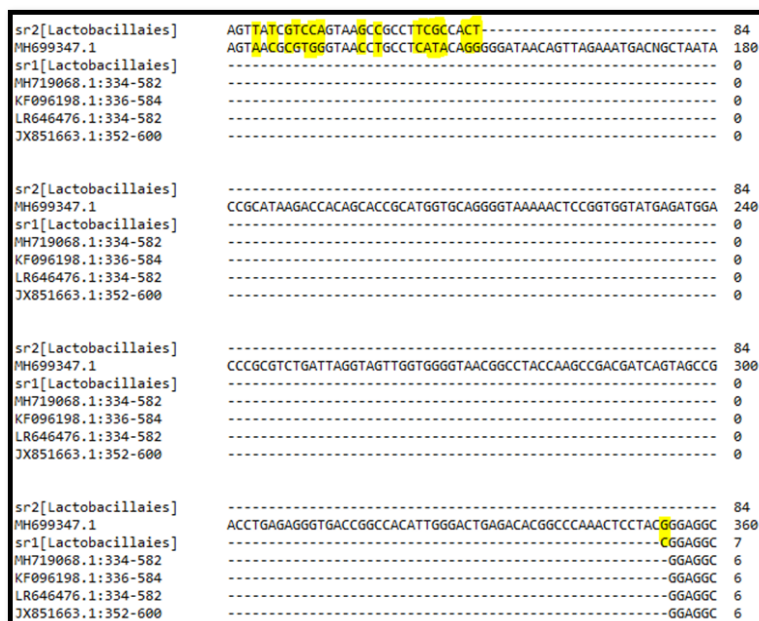
FIGURE 4.1: Similarity between *Lactobacillus* species in Sr2 was checked by using multiple sequence alignment

When these sequence was aligned with other sequences of *Lactobacillaies (Lachnospiraceae bacterium strain AGP2-06-14-05 16S ribosomal RNA gene, partial sequence, Mediterraneibacter faecis strain HBUAS55109 16S ribosomal RNA gene, partial sequence, Uncultured bacterium clone nck220c04c1 16S ribosomal RNA gene, partial sequence, uncultured bacterium partial 16S rRNA gene, and Uncultured bacterium clone PCS406-542 16S ribosomal RNA gene, partial sequence)* the variation of sixteen base were noticed, presented in figure 4.1.

4.5 Phylogenetic Analysis

The phylogenetic tree consists of the 4 major clades i.e. Clade A, Clade B, Clade C and Clade D as shown in figure 4.2. The sequences of 5 bacterial strains were retrieved from the NCBI genbank and the new strain's sequences with the name of sr1 and sr2 were also included in the tree. Phylogenetic analysis using Maximum Parsimony indicates that the sequenced strain seems to independently evolved in Clade D and shows ancestral similarity with *Lachnospiraceae bacterium strain AGP2-14-05.* Multiple sequence alignment also revealed sixteen base pairs variation in sequence when aligned with bacterial sequences.
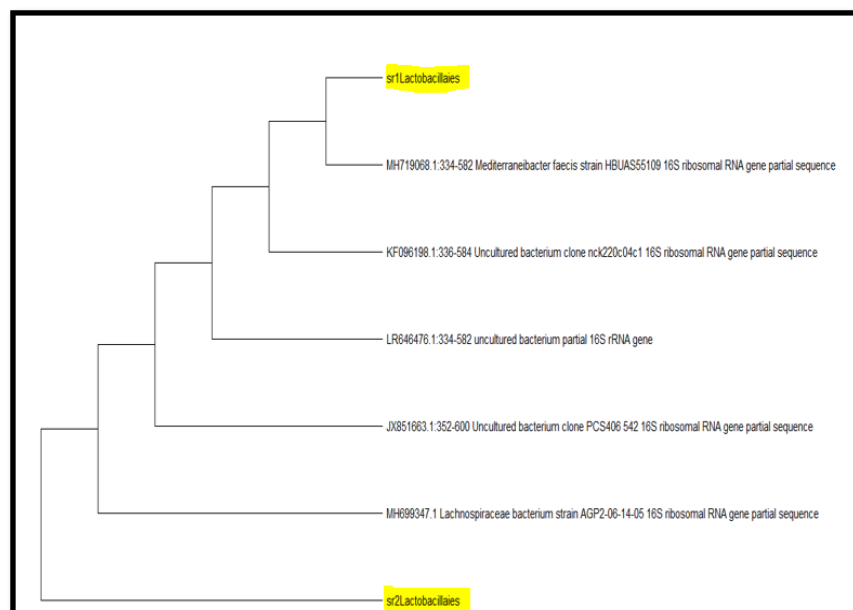
FIGURE 4.2: Phylogentic tree proposed taxonomic division of *Lactobacillius* species

4.6 Taxonomic Bar Plots

4.6.1 Class Taxa Bar Plot

The most prevalent phyla in Sr1 was, *Bacteroidetes* (40%), *Firmicutes of Clostrida* (20%) followed by *Proteobacteria* (15%), *Actinobacteria* (3%) whereas in Sr2 the *Bacteroidetes* (54%), *Fimicutes of Clostrida,* (33%) while *Proteobacteria* (2%) and *Actinobacteria* were 1.5%.While *Firmicutes of Erysipeotrichia* is present about 1% in Sr2 and absent in Sr1, as presented in figure 4.3.
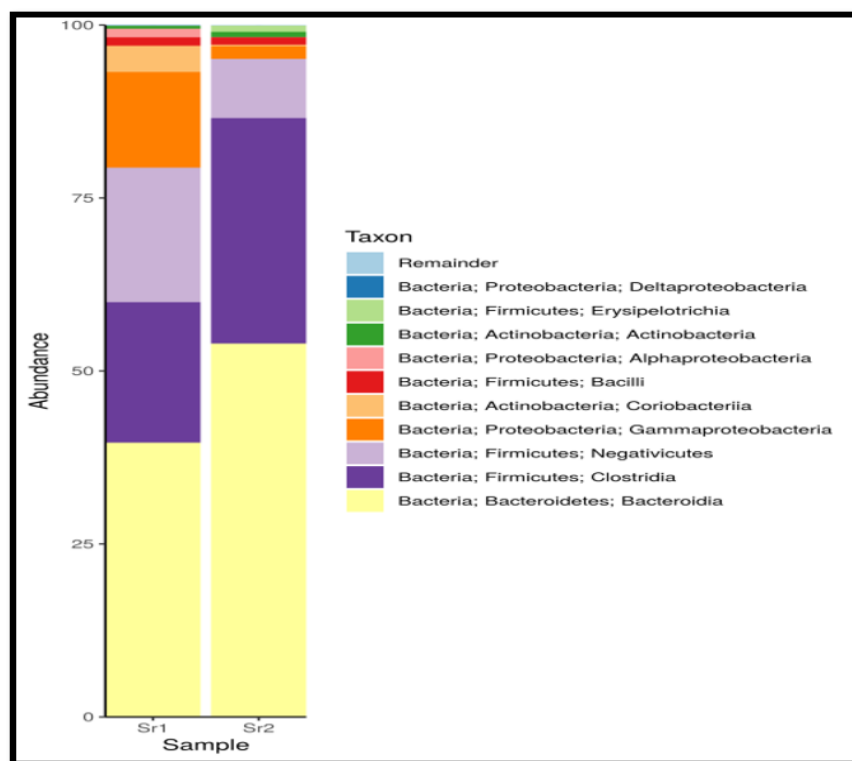
FIGURE 4.3: Taxonomic Class Taxa Bar Plot showing the Classes of bacteria in Sr1 and Sr2 and their abundance

4.6.2 Order Taxa Bar Plot

As shown in 4.4 in order level, *Bacteroidales* (53%) and *Clostridiales* (33%) were present more in Sr2 and less in Sr1 41% and 20% respectively . Whereas *Selenomonadales* was present more extensively in Sr1 (19.5%) as compared to Sr2

(10%). *Enterobacteriales* was more abundant in Sr1 and very less abundant in Sr2 about (1.5%). *Coriobacteriales*(4%), *Betaproteobacteriales* (2.5%) was evident in Sr1 and less in Sr2, 0.5% and 2%. While *Lactobacillales* was 1% in Sr1 and 0.75% in Sr2.
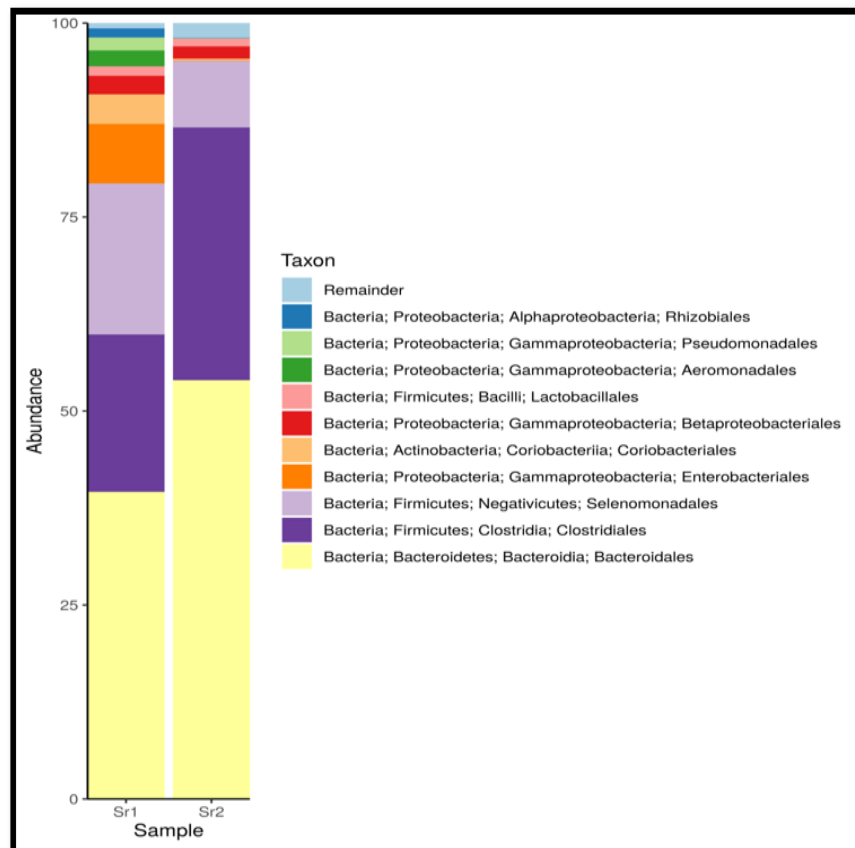
FIGURE 4.4: Taxonomic Order Taxa Bar Plot showing the orders of bacteria in Sr1 and Sr2 and their abundance

4.6.3   Genus Taxa Bar Plot

At genus level *Prevotella* 9 was more abundantly about 36% present in Sr2 and less abundantly 27% present in Sr1. While *Megamonas* was found in (14%) in Sr1 and absent in Sr2. Whereas *Prevotella 2*, *Faecalibcterium*, were more evident in Sr1 about 6% and 8% while in Sr2 they were 5% and 3%. Dialister was present in traces about 9% in Sr2 and absent in Sr1. *Clotridiales* was observed to be 7% in Sr2 and absent in Sr1. *Escherichia-Shigella* were present in percentage of 8% in Sr1 and in traces in Sr2, presented in 4.5.
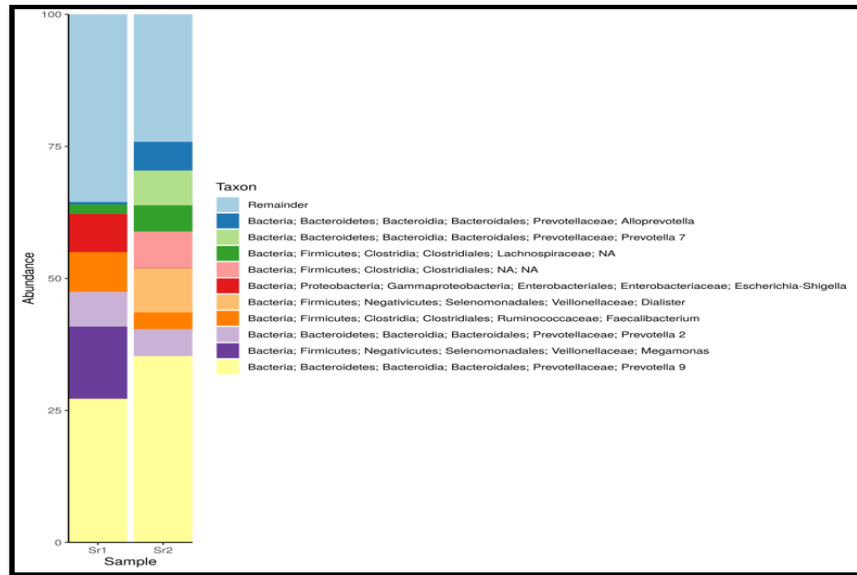
FIGURE 4.5: Taxonomic Order Taxa Bar Plot showing the Genus of bacteria
in Sr1 and Sr2 and their abundance

4.6.4   Species Taxa Bar Plot

In figure 4.15 shows the prevalane of Bacterial species among diabetic and non-
diabetic people. *Escherchia-Shigella* present in Sr1 about 7% and absent in
Sr2. Uncultured bacteria (*Megamonas* 13.5%) present in Sr1 and absent in Sr2.
Provetella 9 were present more in Sr2 (30%) and less in Sr1 (23.5%). *Prevotella
2* is present more (7%) in Sr1 and less (6%) in Sr2. Ambiguous taxa is present
more in Sr1 (40%) and less in Sr2 (30%).
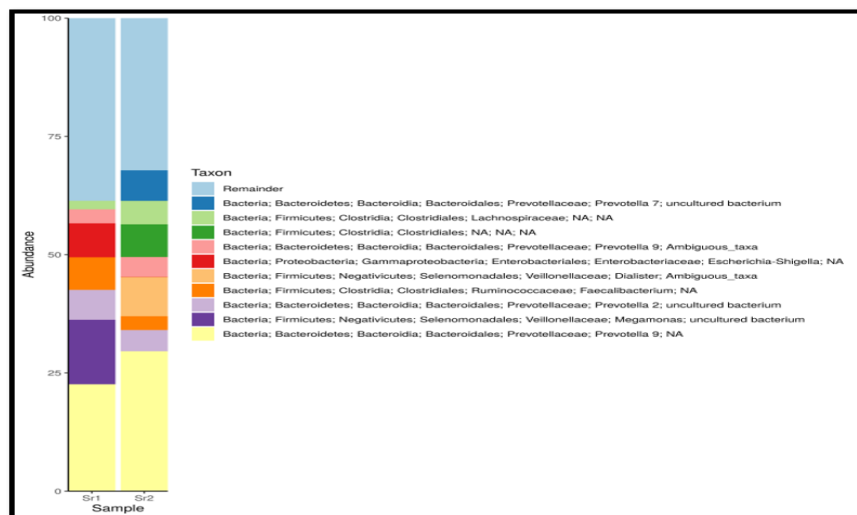
FIGURE 4.6: Taxonomic Order Taxa Bar Plot showing the species of bacteria
in Sr1 and Sr2 and their abundance

4.7   Heat Map

Heat map is the graphical representation of the data for better visualization and understanding of the events within the dataset. Red colour indicates high expression in that region whereas blue colour referred to low expression. As shown in this order level heatmap Sr1 (Control sample) the expression of ordertextit-Bacteroidales, Bacillales, Bifidobacteriales, Campylobcterales, Clostridales and *Pasteurellales* were low whereas the highly expressive orders for *Actinomycetales, Enteobacteriales, Micrococcales, Pseudomonadales, Rhizobiales, Spingomonadales and Xanthomonadales* was observed. *Lactobcillius* belong to order *Lactobacillales*, the expression of *Actinomycetales, Enteobacteriales, Lactobacillales, Micrococcales, Pseudomonadales, Rhizobiales, Spingomonadales* and *Xanthomonadales* was lower in diabetic patients as compared to healthy prople. Similarily, high expression of *Bacteroidales, Bacillales, Bifidobacteriales, Campylobcterales, Clostridales* and *Pasteurellales* sugguested to have association with disease as presented in figure 4.7.
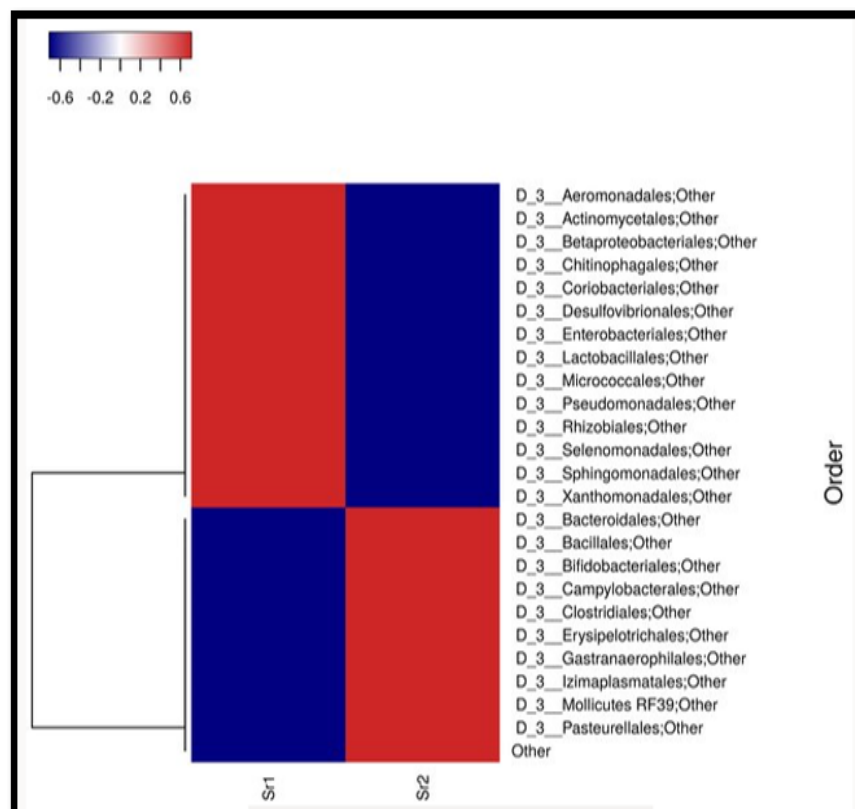
FIGURE 4.7: Heat map of Samples Sr1 (controlled) and Sr2 (diseased)

4.8 Growth on MRS Media

MRS is a selective media that is used for culturing and identification of lactic acid bacteria especially *Lactobacillus*. The growth of *Lactobacillus* is in MRS media and broth is accelarted by the presence of carbon, nitrogen and vitamin source. In MRS broth beef extract, yeast extract and enzymatic extract of animal tissues is used which provides all the essiential nutrients for extended growth of *Lactobacillus*. To provide the media with carbohydrate source dextrose is used that is a fermentable carbohydrate. Ammonium and sodium acetate (act as inhibitory agent) which are used to perform an additional function of providing energy for *Lactobacillius* growth. Maganese sulfate and magnesium sulfate facilitate the media performance by providing cations. Whereas potassium phosphate is a buffering agent. For uptake of nutrients by Lactobacillus a surfactant is used named as polysorbate 80 [120].

Following the experiment when an organism is inoculated in to the broth medium, then those organisms shows maximum growth which are able to ferment dextrose sugar that overcome selective agents and use the nutrients for their growth which is the indication of positive result. Durham tube is used for the differentiation of gas producing and non-gas producing organism. For example this tube is used in tests for indicating difference in *Lactobacillus* and *Leuconostoc* species. Both Samples of diabetic and non-diabetic showed growth of Lactobacillus on MRS media that indicates the presence of *Lactobacillusin* the normal and diseased patients as shown in figure 4.8.

4.9 Biochemical Tests

4.9.1 Microscopic Examination

The shape and microscopic examination of bacterial colonies grown on MRS revealed that the colonies were of white, creamy white or off white in colour whereas
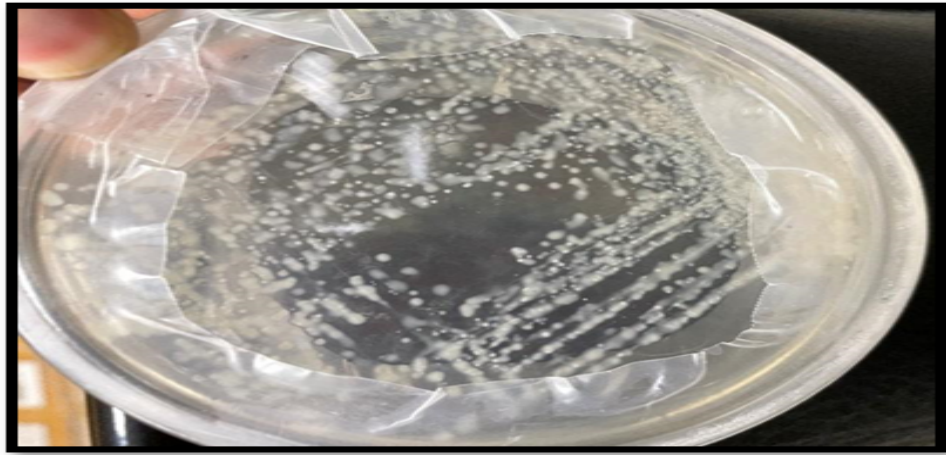
FIGURE 4.8: *Latobacillus* colonies of diabetic and Non-diabetic people grown on MRS media

the shapes of bacteria includes *Bacilli* and *Coccobacilli* as illustated in table 4.1 for diabetic and 4.2 for non-diabetic people.

TABLE 4.1: Biochemical testing of *Lactobacillus* species isolated from diabetic patients

Serial number	Gram Staining	Morphology	Colony Color	Vogus Prosker Test	Oxidase and Catalase Test
1	Gram positive	Bacilli	Creamy white, shiny	-	-
2	Gram positive	Bacilli	Off white	-	-
3	Gram positive	Bacilli	Creamy white	-	-
4	Gram positive	Coccobacilli	White, shiny	-	-
5	Gram positive	Bacilli	White	-	-
6	Gram positive	Bacilli	Off white	-	-

TABLE 4.1: Biochemical testing of *Lactobacillus*species isolated from diabetic patients

Serial number	Gram Staining	Morphology	Colony Color	Vogus Prosker Test	Oxidase and Catalase Test
7	Gram positive	Bacilli	Creamy white	-	-
8	Gram positive	Coccobacilli	White, shiny	-	-
9	Gram positive	Coccobacilli	White	-	-
10	Gram positive	Coccobacilli	Off white	-	-
11	Gram positive	Bacilli	Creamy white, shiny	-	-
12	Gram positive	Bacilli	Creamy white, shiny	-	-
13	Gram positive	Bacilli	Creamy white, shiny	-	-
14	Gram positive	Bacilli	Off white	-	-
15	Gram positive	Bacilli	Off white	-	-
16	Gram positive	Coccobacilli	Off white	-	-
17	Gram positive	Coccobacilli	Off white	-	-

TABLE 4.1: Biochemical testing of *Lactobacillus*species isolated from diabetic patients

Serial number	Gram Staining	Morphology	Colony Color	Vogus Prosker Test	Oxidase and Catalase Test
18	Gram positive	Bacilli	White, shiny	-	-
19	Gram positive	Bacilli	White	-	-
20	Gram positive	Bacilli	Off white	-	-
21	Gram positive	Coccobacilli	White, shiny	-	-
22	Gram positive	Bacilli	White, shiny	-	-
23	Gram positive	Bacilli	Off white	-	-
24	Gram positive	Bacilli	Creamy white	-	-
25	Gram positive	-	Creamy white	-	-
26	Gram positive	Bacilli	Creamy white	-	-
27	Gram positive	Bacilli	Creamy white	-	-
28	Gram positive	Bacilli	Creamy white	-	-
29	Gram positive	Coccobacilli	Creamy white	-	-
30	Gram positive	Coccobacilli	Creamy white	-	-

TABLE 4.1: Biochemical testing of *Lactobacillus*species isolated from diabetic patients

Serial number	Gram Staining	Morphology	Colony Color	Vogus Prosker Test	Oxidase and Catalase Test
31	Gram positive	Coccobacilli	Creamy white	-	-
32	Gram positive	Coccobacilli	Off white	-	-
33	Gram positive	Coccobacilli	Off white	-	-
34	Gram positive	Bacilli	White, shiny	-	-
35	Gram positive	Bacilli	Off white	-	-
36	Gram positive	Bacilli	White, shiny	-	-
37	Gram positive	Bacilli	White, shiny	-	-
38	Gram positive	Bacilli	White, shiny	-	-
39	Gram positive	Bacilli	White, shiny	-	-
40	Gram positive	Bacilli	White, shiny	-	-
41	Gram positive	Bacilli	White, shiny	-	-
42	Gram positive	Bacilli	Creamy white, shiny	-	-

Table 4.1: Biochemical testing of *Lactobacillus*species isolated from diabetic patients

Serial number	Gram Staining	Morphology	Colony Color	Vogus Prosker Test	Oxidase and Catalase Test
43	Gram positive	Bacilli	Off white	-	-
44	Gram positive	Bacilli	Creamy white	-	-
45	Gram positive	Bacilli	White, shiny	-	-
46	Gram positive	Bacilli	Creamy white, shiny	-	-
47	Gram positive	Bacilli	Off white	-	-
48	Gram positive	Bacilli	Creamy white, shiny	-	-
49	Gram positive	Bacilli	Creamy white, shiny	-	-
50	Gram positive	Bacilli	Creamy white, shiny	-	-

Table 4.2: Biochemical testing of *Lactobacillus* species isolated from non-diabetic people

Serial number	Gram Staining	Morphology	Colony Color	Catalase, Oxidase and Vogus Prosker Test
1	Gram positive	Bacilli	Off white	-

Table 4.2: Biochemical testing of *Lactobacillus* species isolated from non-diabetic people

Serial number	Gram Staining	Morphology	Colony Color	Catalase, Oxidase and Vogus Prosker Test
2	Gram positive	Bacilli	Creamy white	-
3	Gram positive	Coccobacilli	White, shiny	-
4	Gram positive	Coccobacilli	Creamy white, shiny	-
5	Gram positive	Coccobacilli	Off white	-
6	Gram positive	Bacilli	Off white	-
7	Gram positive	Bacilli	Off white	-
8	Gram positive	Bacilli	White, shiny	-
9	Gram positive	Bacilli	Off white	-
10	Gram positive	Bacilli	White, shiny	-
11	Gram positive	Coccobacilli	Off white	-
12	Gram positive	Coccobacilli	Off white	-
13	Gram positive	Bacilli	White, shiny	-

TABLE 4.2: Biochemical testing of *Lactobacillus* species isolated from non-diabetic people

Serial number	Gram Staining	Morphology	Colony Color	Catalase, Oxidase and Vogus Prosker Test
14	Gram positive	Bacilli	Off white	-
15	Gram positive	Bacilli	White, shiny	-
16	Gram positive	Bacilli	White, shiny	-
17	Gram positive	Bacilli	White, shiny	-
18	Gram positive	Bacilli	Creamy white	-
19	Gram positive	Bacilli	Creamy white	-
20	Gram positive	Bacilli	Creamy white	-
21	Gram positive	Coccobacilli	Creamy white	-
22	Gram positive	Coccobacilli	Creamy white	-
23	Gram positive	Coccobacilli	Creamy white	-
24	Gram positive	Coccobacilli	Creamy white	-
25	Gram positive	Bacilli	Creamy white	-

TABLE 4.2: Biochemical testing of *Lactobacillus* species isolated from non-diabetic people

Serial number	Gram Staining	Morphology	Colony Color	Catalase, Oxidase and Vogus Prosker Test
26	Gram positive	Bacilli	Off white	-
27	Gram positive	Bacilli	Off white	-
28	Gram positive	Bacilli	Off white	-
29	Gram positive	Bacilli	Off white	-
30	Gram positive	Bacilli	Off white	-
31	Gram positive	Bacilli	Off white	-
32	Gram positive	Bacilli	Off white	-
33	Gram positive	Bacilli	Off white	-
34	Gram positive	Bacilli	Off white	-
35	Gram positive	Bacilli	Off white	-
36	Gram positive	Bacilli	Off white	-
37	Gram positive	Coccobacilli	Off white	-

TABLE 4.2: Biochemical testing of *Lactobacillus* species isolated from non-diabetic people

Serial number	Gram Staining	Morphology	Colony Color	Catalase, Oxidase and Vogus Prosker Test
38	Gram positive	Bacilli	Off white	-
39	Gram positive	Bacilli	Off white	-
40	Gram positive	Bacilli	White, shiny	-
41	Gram positive	Bacilli	Off white	-
42	Gram positive	Bacilli	White, shiny	-
43	Gram positive	Coccobacilli	Off white	-
44	Gram positive	Coccobacilli	Off white	-
45	Gram positive	Bacilli	White, shiny	-
46	Gram positive	Bacilli	Off white	-
47	Gram positive	Bacilli	White, shiny	-
48	Gram positive	Bacilli	White, shiny	-
49	Gram positive	Bacilli	Off white	-
50	Gram positive	Bacilli	Off white	-

+= Positive reaction of all isolates, -= Negative reaction.

Out of 50 diabetic samples 37 *Lactobacillus* species were bacilli and 13 were coccobacilli. Among all the bacilli, 9 were off white, 8 were white and 16 were creamy white, as mentioned in table 4.1. On the basis of colonial morphology that was observed, the white circular, large, smooth, glistering isolates are suspected to be *L. acidophilusand L. rhamnosus.* The creamy white, smooth, mucoid, convex would be *L. casei.* Similarly, among coccobacilli 5 were off white, 4 were white and 5 were creamy white. Out of 50 non-diabetic samples, 38 isolates were bacilli and 12 were cocobacilli. In non-diabetic study subjects, out of all the bacilli samples, 20 were off white, 10 were white and shiny. Similarly, among coccobacilli in nondiabetic study subjects, 4 were off white, 1 white and 5 were creamy white. As described in Table 4.2.

4.10   Gram Staining

Gram staining of the bacterial colonies showed that they were gram positive bacteria. *Lactobacillus* is a gram positive bacteria which when viewed under microscope gives purple color due to the existence of a dense peptidoglycan cover in the cell wall which retains the purple color of the crystal violet during the process of decolorization as described in Figure 4.9.
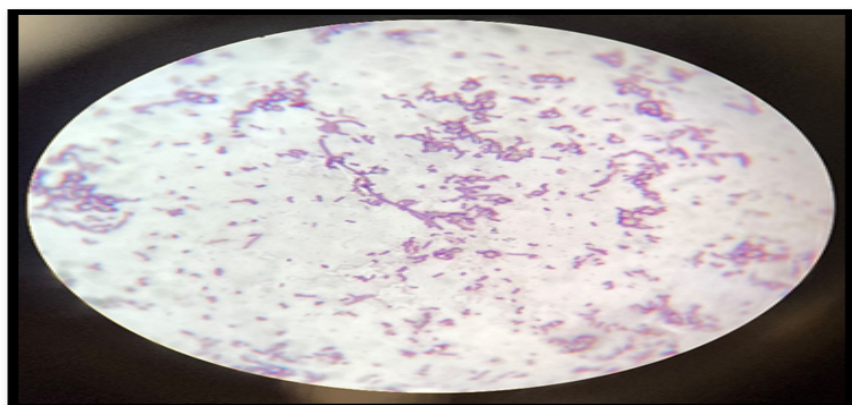
FIGURE 4.9: Gram staining of isolates obtained from diabetic and non-diabetic people

4.11   Oxidase Test

For carrying out oxidase test, Kovacs reagent was used. A piece of filter paper was soaked in the reagent and left to dry out. Using a sterilized wire loop, the isolated bacterial colonies were transferred from the plates to the filter paper. After, 2 minutes the results were noted.This is very important test to be carried out. An Intracellular oxidase enzyme is produce by cytochrome containing organisms. The catalyization of Cytochrome c is done by of this oxidase enzyme.During experiment when reagent turns purple or blue, it indicate the presences of cytochorme c as a part of their respiratory chain these label as the positive result. Whereas if the reagent is not oxidased, appear colourless with in the test limits, it indicates the absence of cytochrome c as a part of their respiratory chain [122]. All samples of both non-diabetic and diabetic people cultured on MRS media were negative for oxidase test as no colour change was observed as evident from Figure 4.10.

FIGURE 4.10: Oxidase test result of isolates separated from diabetic and non-diabetic individuals.

4.12   Catalase Test

Catalase is an enzyme which catalyses the discharge of oxygen from hydrogen peroxide(H_2O_2), catalase test indicates the presence of catalase. This test is specifically used to distinguish between catalase producing bacteria (*staphylococci*) and

non-catalase producing bacteria(*streptococci*). For detection of catalase in anaerobes15% H_2O_2 is used while for routine culture routine 3% H_2O_2 is used. The catalase enzyme facilitates the collapse of H_2O_2 into oxygen and water. In experiment presences of catalase in provided bacterial isolate is evident by bubble formation when a minor inoculum is familiarized into H2O2, the speedyembellishment of oxygen bubbles happens [121]. While weakor no bubble formation indicate absences of catalase.Diabetic and non-diabetic samples grown on MRS media were negative as indicated in figure 4.11. A catalase test was used for *Lactobacillus* identification which is gram positive bacteria.
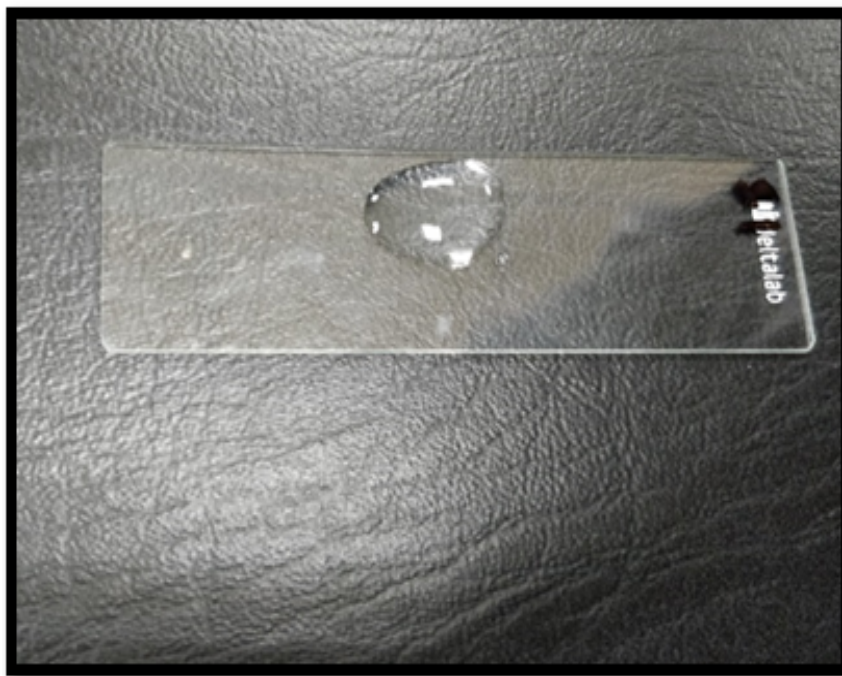
FIGURE 4.11: Catalase result of isolates performed to check presences of *Lactocillilus* in diabetic and non-diabetic samples

4.13 Voges Proskure Test

To find out whether an organism produces acetylmethyl carbinol from glucose fermentation, the Voges- Proskauer (VP) test is used. Acetylmethyl carbinol, if present, undergoes diacetylation in the presence of -naphthol, strong alkali (40% KOH), and oxygen from the atmosphere. Barritt discovered that the -naphthol

acts as a colour intensifier and that it must be introduced first even though it was not a part of the original technique [123]. The peptones of the broth's diacetyl- and quanidine-containing molecules then condense to create a pinkish-red polymer. It was negative for both diabetic and non-diabetic samples cultured on MRS media (shown in figure 4.12)

FIGURE 4.12: VP test results of isolatesof diabetic and non-diabetic peoples

4.14   Tests for Anti-Diabetic Drug Resistance

Anti-diabetic sensitivity test was performed in order to check whether the growth of Lactobacillus species are affected by anti-diabetic drugs (Metformin, Acarbose, Sitagliptin and Metformin+Sitagliptin). No zone of inhibition was observed for any of anti-diabetic drug. The association of drugs with the gut microbiota is thought to catch much interest [124].
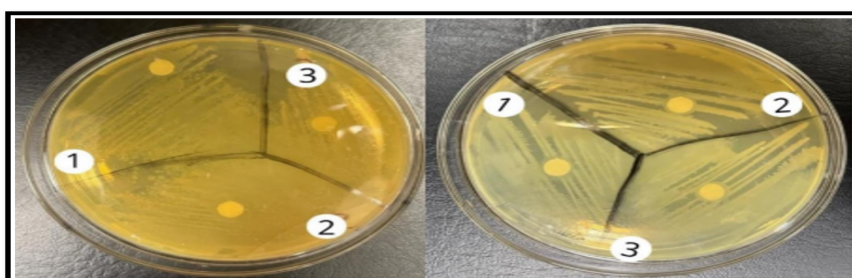
FIGURE 4.13: Results of Metformin sensitivity on diabetic (right) and non-diabetic (left) samples.

Studies have shown that abundance of genus *Lactobacillus* has relatively increased by Metformin and sitagliptin as compare to other genera. Metformin 50 increases the GLP-1 plasma levels and also enhances GLP-1 expression in the pancreas islets. Metformin has positive effects on SCFA (short chain fatty acids) producing bacteria which include *Bacteroides*, and *Lactobacillus*. Metformin plays a role in glucose intake regulation. In previous studies, metformin was also studied to alter the gut microbiota composition but it was also dependent on the diet of the subjects [125]. Results of Metformin sensitivity on diabetic (right) and non-diabetic (left)shown on figure 4.13.

Sitagliptin belongs to a class of incretin-based drugs. Sitagliptin has been studied to bring the gut microbiota levels to normal in diabetic subjects at the phylum level. The half-life of incretin-based peptides is very short and are cleared by renal filtration. The results of sitagliptin were shown in figure 4.14.
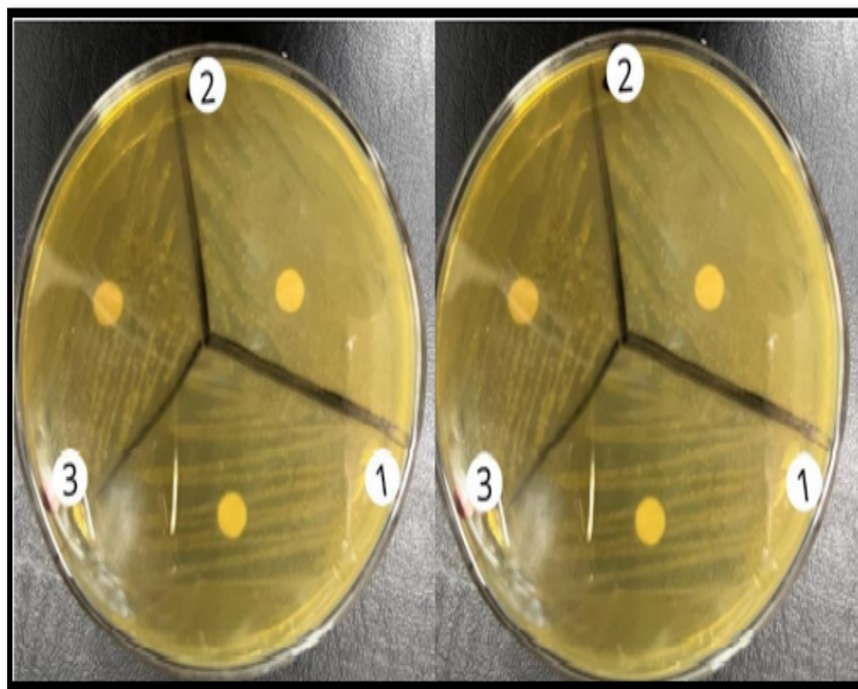
FIGURE 4.14: Results of Sitagliptin sensitivity on diabetic (right) and non-diabetic (left) 51 samples. 1: 25mg drug with 12.5ml water. 2: 50mg drug with 25ml water 3: 100mg drug with 50ml water.

Acarbose belongs to drug class alpha-glucosidase inhibitors and alter the nutrient source of bacteria by the reduction of postprandial hyperglycemia and in the small intestines delay the digestion of carbohydrates. Previous studies have shown an

increased relative abundance with the decreased diversity of the beneficial bacteria by acarbose in the type 2 diabetic patients. Acarbose reduces the concentration of propionate in the body which is linked with reduction in starch processing. Acarbose enhances the abundance of both butyrate-producing and starch fermenting bacteria such as *Lactobacillus* species. Acabose results are shown in figure 4.15.
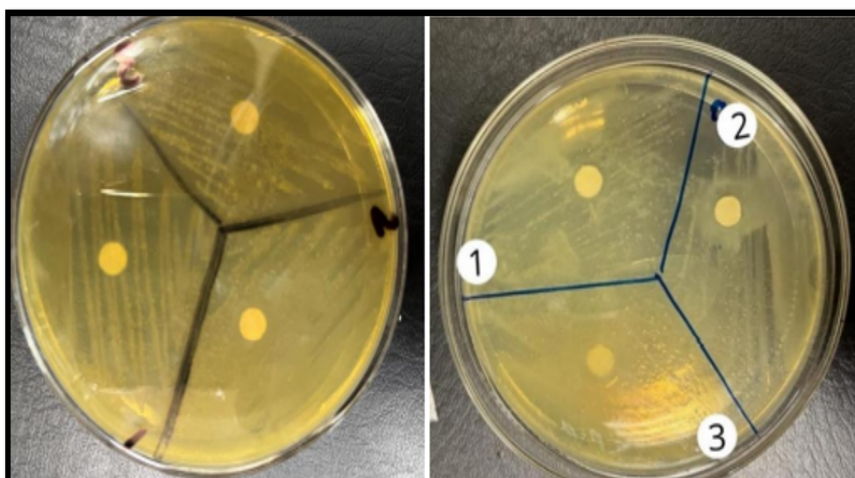
FIGURE 4.15: Results of Acarbose sensitivity on diabetic (right) and non-diabetic (left) samples. 1: 25 mg drug with 12.5 ml water. 2: 50 mg drug with 25ml water. 3: 100 mg drug with 50ml water

No zone of inhibition was observed for Metformin+Sitagliptin as shown in figure 4.16. It is observed that the anti-diabetic drugs can modify the gut microbiota and ultimately improves the diabetes. The monotherapy of Metformin, sitagliptin and acarbose decreases the blood glucose levels and effectively used for treatment.
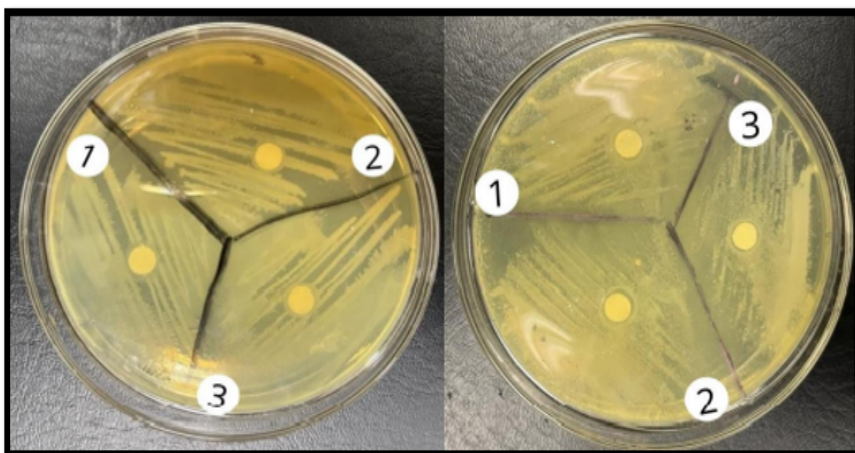

FIGURE 4.16: Result ofMetformin +Sitagliptin sensitivity on diabetic (right) and non-diabetic (left) samples. 275mg with 137.5ml water, 550 mg with 275ml water and 1100 mg with 550ml water.

4.15 Discussion

Insulin resistance associated with obesity is the main cause of type 2 diabetes, a metabolic condition. Infection, emotional stress, and a genetic predisposition to diabetes are among additional contributing causes [126]. Inflammatory mediators including tumour necrosis factor and interleukins are abnormally expressed and produced in both obesity and diabetes, which are both defined as states of chronic low-grade inflammation [127]. Recent studies have demonstrated a link among the arrangement of the intestinal microbiota and metabolic diseases like obesity and diabetes using large-scale 16S rRNA gene sequencing as well as more specialised methods based on quantitative real time PCR (qPCR) and fluorescent in situ hybridization (FISH) [128]. For instance, in prebiotic-treated mice, levels of *Bifidobacterium* expressively and favourably linked with better glucose tolerance and low-grade inflammation [128]. Additionally, it was noted that the more *Bacteroides* species were associated with the development of type 1 diabetes in rats. According to a theory put up by researchers, the development of insulin resistance is a result of the host's increased hepatic production of triglycerides and increased gastrointestinal absorption of monosaccharides by the gut microbiota [129]. Evidence from numerous research in people and mouse models showed that a rise in body mass was linked to a higher percentage of *Firmicutes* and a relatively lower number of *Bacteroidetes* [130]. *Firmicutes* to *Bacterodetes* ratios in obese human adults as compared to lean controls. There is no evidence to support a connection between the proportion of *Bacteroidetes* and *Firmicutes* and human obesity, according to a different study employing weight loss programmes [131].

As a result, it is still unclear what the microbiome of an obese person looks like, and additional research is required to clarify how the gut microbiota and metabolic illnesses [132]. Variations in the intestinal microbiota, inflammation, and disruption of the intestinal barrier are frequently seen in obesity and T2D. A common feature of T2D and obesity is a persistent, low-grade inflammatory response; this systemic inflammatory response is also regarded to be the primary driver of insulin resistance [133]. Previous studies in mice models have shown that the gut

microbiota is to blame for the heightened inflammatory response in obese human adults as related to lean controls in the respective *Firmicutes* to *Bacterodetes* ratios. Another study with weight loss programmes initiates no evidence to suggest a link between the proportion of *Bacteroidetes* and *Firmicutes* and human obesity [134]. A metagenomic research revealed that there are frequently significant differences between the intestinal microflora of people with T2D and healthy people, and that the loss of butyrate-producing bacteria may be the root source of poor glucose metabolism [135]. When the gut microbiota is altered by outside factors like nutrition, this can result in intestine microbial metabolite dysregulation and secretory alterations, which can set off a number of potential pathways that might contribute to insulin resistance and diabetes [136]. In addition, by altering how they react to food components, gut microbiota might influence metabolism and the possible risk of developing diabetes [137]. An immunological and low-grade inflammatory response in which the gut microbiota and its metabolites are important factors. Studies have shown that patients with T2D and non-diabetic patients have very different oral microbiotas [138]. Oral microbial indicators for T2D screening, diagnosis, and prediction have been discovered [139]. Recently, scientists offered a potential explanation for how diabetes affects the likelihood and severity of tooth loss. Diabetes may alter the composition of oral microorganisms, and experiments using germ-free mice revealed that the oral microbiota of diabetic mice was additional pathogenic [140].

Chapter 5

Conclusions and Recommendations

Metabolic diseases including obesity and diabetes have become a social problem for countries in all over the world. Type 2 diabetes mellitus (T2DM) is more widespread type of diabetes. In type 2 diabetic patients take oral medication or insulin to maintain blood glucose level. Human gut microbiota has proven to be associated with various metabolic syndromes such as T2D. Diabetic patients have shown variation in population of microbiome as compared to the healthy people. So keeping in view, the first objective was to check the variations of Lactobacillus in diabetic and healthy people. It is concluded that 100 samples taken out of which 50 were of diabetic patients and 50 were of non-diabetic patients. The *Lactobacillus* species were cultured on nutrient agar. To evaluated the difference between diabetic and healthy people metgenomics was performed. To ensure the presences of these bacterial strains gene sequence analysis was done. The *Lactobacillus* species in non-diabetic samples are in *Protobacteria* 2%, order *Lactobacillales* is 0.75%. While at genus level the genus *Provetalla 9* is 27%. The most prevelent class are *Bacteroidetes* 40% and *Firmicutes* 20% in control sample. In representation of heat map the expression of *Lactobacillus* was greater in control sample. Korona plot also indicate the great presences of *Lactobacillus*. Then the bacteria were cultured on MRS media. Biochemical tests of the isolates confirmed the presence

of *Lactobacillus* species. The bacteria were gram positive bacilli and coccobacilli. Second goal was to chemically characterize or confirm the *Lactobacillus*. The catalase test for the isolates was negative which meant that they lacked the presence of catalase enzyme. The Oxidase test for the isolates was negative which meant the absence of cytochrome oxidase. The isolates also tested negative for Voges-Proskauer test. To achieve third goal antidiabetic sensitivity test was performed to check whether the growth of Lactobacillus spp. is affected by Anti-diabetic drugs (Metformin, Acarbose, Sitagliptin, and Metformin+Sitagliptin). No zone of inhibition was observed for any of the antidiabetic drugs, which shows that anti-diabetic drugs do no inhibit the growth of *Lactobacillus* species.

1. The samples have been collected from Islamabad city. In future irrespective of ethnicity samples must include wider aspect of population including ethnicities from different areas of Pakistan as different results have been reported from different populations of the world with respect to mircobiome of diabetic and non-diabetic people.

2. Effect of anti-diabetic drugs must be evaluated other species of bacteria that shows significant variation in healthy and diabetic people.

3. Factors like age, gender and food must also be study for their association with diabetic and healthy people for microbiome.

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TABLE 5.1: Decreased ↓, increased ↑, Blood glucose in fasting FBG, concentration of insulin IC, insulin resistant IR, tumor necrosis factor-α TNF-α, IL-1β interleukin1β, superoxide dismutase SOD, homeostasis model assessment-estimated insulin resistance HOMA-IR, sensitivity index ISI, serum levels deoxycholic acid DCA, glycosylated hemoglobin A1c, high density of lipoprotein cholesterol HDL-C, glutathione peroxidase GPx, total antioxidant capacity TAC.

Probiotic	Subject	Dose/duration	Treatment with Probiotic
Bifidobacterium,			
Lactobacillus,	53 individuals having diabetes	Lactobacillus, Lactococcus dose is 6 × 1010 CFU g-1.	IL-1 β HOMA↓, TNF-α↓,
Lactococcus,	2 type		HbA1c, IL-6↓
Propionibacterium			
Lactobacillus casei	20 individuals having diabetes 2 type	8 weeks duration of 1 × 108 CFU.	FBG↓, IC↓
Lactobacillus reuteri	46 individuals having diabetes	L. reuteri DSM dose given is 17938 1010 CFU g. For duration of 12 weeks.	DCA↑, ISI↑,
DSM 17938	2 type		

Table 5.1 continued from previous page

Viable strains,			
7 in number of	30 individuals	L. acidophilus dose of (2.0 × 109 CFU)	FPG↓,
	having diabetes	L. casei dose of (7.0 × 109 CFU)	
Lactobacillus,	2 type	L. rhamnnosus dose of (1.50 × 109 CFU)	HDL-C↑
.			
	68 individuals	L. casei dose of (4.0× 1010	Bowel disbiosis is partially
Lactobacillus casei	having diabetes	CFU)- in fermented milk for	improved in type 2 diabetes
	2 type	duration of 16 weeks.	