Determining the Reversal potential of fecal microbiota (FM) as a part of fecal microbiota transplantation (FMT) for the treatment of diet induced diabetes

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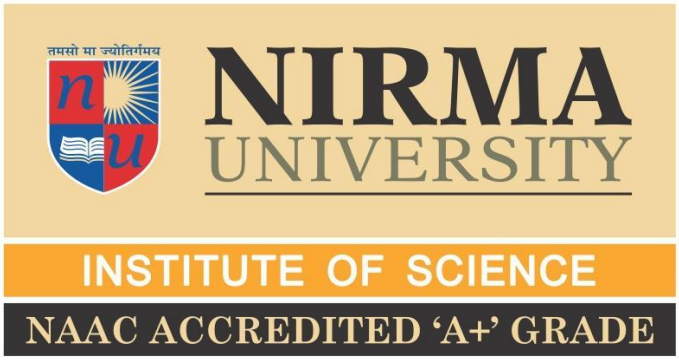

Under the guidance of

Dr. Sriram Seshadri

DEDICATION

This thesis is lovingly dedicated to our rat models who sacrifice their life for our dissertation.

NIRMA UNIVERSITY
INSTITUTE OF SCIENCE
NAAC ACCREDITED 'A+' GRADE

CERTIFICATE

This is to certify that the thesis entitled **"Determining the Reversal potential of fecal microbiota (FM) as a part of fecal microbiota transplantation (FMT) for the treatment of diet induced diabetes"** submitted to the Institute of Science, Nirma University in partial fulfilment of the requirement for the award of degree of M.Sc. (Microbiology / Biotechnology), is a faithful record of bonafied research work carried out by **Anshu Shrivastav (20MMB032), Amrita Menon (20MBT002), Kashyapi Joshi (20MBT019) and Mansi Thakkar (20MBT055)** under the guidance of **Dr. Sriram Seshadri**. No part of the thesis has been submitted for any other degree or diploma.

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CONTENT

LIST OF ABBREVIATIONS

- **ANOVA** Analysis of variance
- **ASD** Autism spectrum disorder
- **AUC** Area Under (the) Curve
- **CB1** Cannabinoid 1 receptor
- **CDI** Clostridium difficile infection
- **CFU** Colony forming unit
- **CPCSEA** Committee for the Purpose of Control and Supervision of Experiments on Animals
- **CRC** Colorectal cancer
- **DC** Diabetic Control
- **DNA** Deoxyribonucleic acid
- **F** Forward
- **FDA** Food and Drug Administration
- **FM** Fecal microbiota
- **FMT** Fecal Microbiota Transplantation
- **FOS** Fructooligosaccharides
- **GI** Gastro-Intestinal
- **GIT** Gastro-Intestinal Tract
- **GOS** Galactooligosaccharides
- **H&E** Hematoxylin and Eosin
- **HDL** High-Density Lipoprotein
- **HPLC** High-performance liquid chromatography
- **HSFD** High Sugar Fat Diet
- **IBD** Inflammatory Bowel Diseases
- **IBS** Irritable Bowel Syndrome
- **IR** Insulin Resistance
- **LDL** Low-Density lipoprotein
- **LI** Large intestine
- **LPS** Lipopolysaccharides
- **NC** Normal Control
- **NF-kB** Nuclear Factor kappa B
- **NK** Natural killer cells
- **OGTT** Oral glucose tolerance test
- **PBS** Phosphate Buffer Saline
- **PCR** Polymerase chain reaction
- **R** Reverse
- **RNA** Ribonucleic acid
- **RO** Reverse osmosis
- **RT** Room temperature
- **RT-PCR** Reverse transcription polymerase chain reaction
- **SCFA** Short-chain fatty acid

- **SE** Standard error
- **SEM** Standard error of the mean
- **SGOT** Serum Glutamic Oxaloacetic Transaminase
- **SGPT** Serum Glutamic Pyruvic Transaminase
- **SI** Small intestine
- **T2DM** Type 2 Diabetes Mellitus
- **TG** Triglycerides
- **TLR** Toll like receptor
- **Tm** Melting temperature
- **XOS** Xylooligosaccharides

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ABSTRACT

ABSTRACT

Human body contains millions of microorganisms and these microorganisms play a vital role in the human body. From that, numerous bacterial species colonize the human gastrointestinal (GI) tract and the functions in aiding digestion, nutritional provision, colonic epithelial maturation, and pathogen protection.

The human gut microbiome differs from person to person and is relatively stable and resilient over time; however, factors such as diet, stress, can change the composition. The altered composition of these microorganisms in the body is called 'gut dysbiosis'.

One of the most common lifestyle diseases is type 2 diabetes mellitus (T2DM); it is due to insulin resistance which leads to abnormal glucose regulation or hyperglycemia. In some cases, the alteration in the gut microbiome is observed as well.

The most common drug used for the treatment of T2DM is metformin that has many advantages including its neutral effect, reduction in body weight, and cardio protective antibiotic. Apart from the benefits it is reported that the drug also has some downsides, it tends to alter the gut microbiota and increases the LPS content.

To overcome the disadvantage, FMT is being used as a treatment strategy in this study. Fecal Microbiota Transplantation (FMT) is a therapeutic procedure of transplanting fecal bacteria from healthy donors into diseased recipients.

Male wistar strain rats (Protocol number-IS/PHD/27/2020/032) were used, with the help of a high sugar high fat diet, T2DM was induced in the rats after the animal grouping. Biochemical test- oral glucose tolerance test (OGTT) was performed to check the induction of diabetes and the reversal before autopsy.

The treatment phase was 12 weeks. Prior to the treatment omeprazole (20 mg/kg) was given; For the treatment FMT (100 mg/kg); crude fecal was collected from the healthy donor; processed and administered via oral gavage to the rats. Metformin (100 mg/kg) was used as a standard drug for administration in another group of diabetic rats.

After autopsy, the collected blood and tissues were used for serum biochemical analysis and gene expression studies, respectively. And from the fecal sample collected previously, colony forming units (CFU) count, short chain fatty acid (SCFA) analysis by HPLC were carried out.

To conclude, the FM can be used for the treatment of gut dysbiosis caused as a part of any metabolic disorder.

INTRODUCTION

INTRODUCTION

Human body contains millions of microorganisms. These organisms serve a critical part in maintaining our internal body wellness. These microbes vary from location, the food we eat and also by the stress we are facing. (Gupta *et al*., 2016) It has also been reported that these microorganisms can improve our metabolism, immune functioning and also helps in the proper functioning of our body.

Many of these species are not culturable and hence 16s RNA sequencing is done to identify them. (Gupta *et al*., 2016) Out of these some species like Bacteroides, Bifidobacterium and Lactobacillus have the above advantages. There are many a times when our gut microbiome changes or the equilibrium of the gut microbiota varies this can be due to the use of antibiotic or it can also occur under certain diseased condition apart from this due to the same change disease can occur this whole condition is referred to as 'Gut Dysbiosis'. (Zhu *et al*., 2020) Well known disease where the gut dysbiosis occurs is cancer, type II diabetes mellitus, autism and many other conditions. (Zhu *et al*., 2020) To restore the alteration, medications such as antibiotics are used. Other treatment strategies include the use of pre and probiotics, which are live microbes that are given to restore the altered gut microbiome.

Apart from these, another important method for the restoration process would be the transplantation of feces from a healthy person to one of a diseased people that is known as 'Fecal Microbiota Transplantation.' (Zhu *et al*., 2020) The fecal matter collected is stored or directly used and given to the recipient either in normal water, saline or with PBS. The prepared solution is either administered using nasogastric tube, nasojejunal tube or orally. The most commonly used technique is orally as it is less destructive. The above technique was found very successful on the treatment of infection caused by *Clostridrium difficle* the success rate was around 90% other disease treated via this method was crohn ulcerative, colon irritation. (Chanyi *et al*., 2017)

The recent studies showed that the are many diseases that occurred due to lifestyle change of these one of them is type II diabetes this condition is also called as hyperglycemia where the body makes insulin but it doesn't respond to it, as glucose transporters are unable to transfer

the glucose which leads to insulin resistance and this is the world's seventh biggest cause of death as early detection of this condition is not possible. The treatment is also considered costlier and it was noted that during earlier days it occurred above the age of 40 but now this trend has changed and now it is common in children due to lifestyle changes or some other reasons. (Kharroubi *et al*., 2015) As, T2DM is a condition where insulin is produced by the beta cells of pancreas but is not absorbed into the cell or the cell is not able to utilize the produced insulin and due to this the glucose present in the body is not absorbed which leads to the accumulation of glucose. (Kharroubi *et al*., 2015) The accumulation of glucose also causes obesity and high blood pressure, high LDL, low HDL. Along with this T2DM also causes gut dysbiosis.

A healthy gut always contains beneficial bacteria that helps in the process of digestion, solubilizing toxins and producing some vital compounds for us. In the case of T2DM the intestinal composition changes that may lead to inflammation and that is further may be one of the reasons for insulin resistance change in microbial composition can also lead to the expression of inflammatory signals and can cause oxidative stress and decreased production of minerals and vitamins in our body. (Kharroubi *et al*., 2015; Salgaco *et al*., 2019) To cure the T2DM the most common drug administered is Metformin but due to its side effects that was noted an alternate need to be used and this alternate was FMT. (Elbere *et al*., 2018) It can be more promising as they tend to restore the dysbiosis therefore in our study we are using FMT as one of the treatment strategies for treating diet induced diabetes (T2DM) which will be compared with the other group treated with the standard drug metformin. (Salgaco *et al*., 2019; Elbere *et al*., 2018)

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Microbiome (Function & Diversity)

Humans are not sterile entities starting from the exterior skin to the entire body; they contain microscopic life; these microscopic lives are called the "second genome". These microscopic life aids in many processes like digestion, providing nutrition, colonic epithelial maturation, host immunity and regulation of neuropsychological behaviors. (Wang *et al*., 2018; Bokoliya *et al*., 2021)

These microbes also act as the primary line of defense and protect the intestinal mucosa. The main compositions of the human gut are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. (Zakria *et al.*, 2020) Most of all microorganisms present in the human gut are anaerobes. To identify these, many techniques are used which are from FISH to microarray.

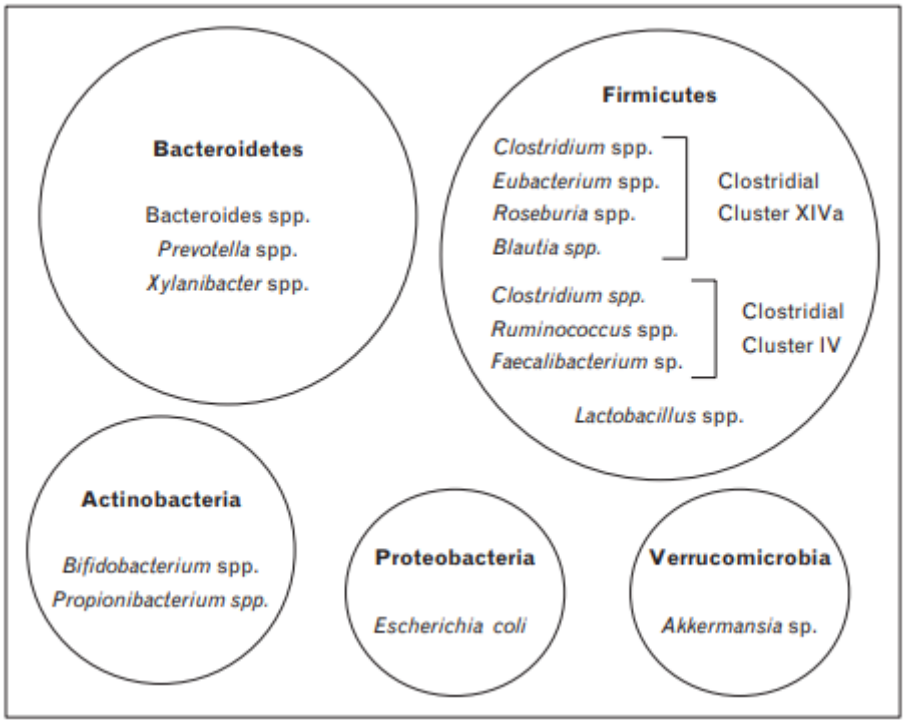

Figure 1: Predominant species of human colon. (Chass *et al*., 2013)

The gut microbiota is different in the different individual microbiome of an infant is different than of the adult and in adult also the micro biotic content of male and female will be different also as we age the composition of the gut alters and accounts for various disease associated with gut in old age people, apart from this gut microbiota varies from person to person this

mainly depends on the diet we eat, the physiology conditions and also the stress conditions. Sometimes due to the medication the microbial composition tends to change. (Lozupone *et al*., 2012)

They main aim of the gut microflora is to breakdown complex substances into smaller pieces that include the metabolism of carbohydrates, lipids and sugars these are converted into SCFA of which butyrate is absorbed by colonic cells while acetate and propionate is transported into liver and heart for gluconeogenesis and oxidation, respectively.

The bacterial composition is usually Firmicutes and Bacteroides bacteria and there is always an equilibrium between these two in a healthy gut. Usually, alternation in these two leads to the inflammation of the gut that in turn can be associated with many diseases like *clostridium difficile* (CD) infection, Crohn ulcerative. (Wang *et al*., 2018) Most of all the diseases associated with dysbiosis that can be cured by taking substances that can re-establish gut microflora.

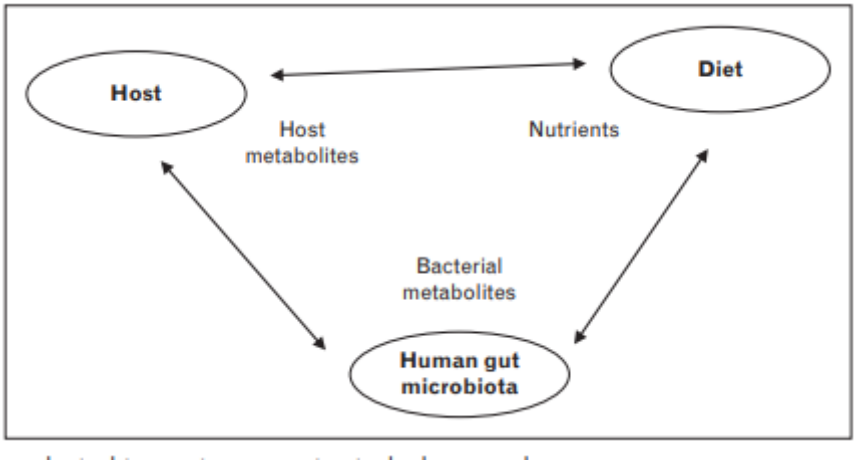

Figure 2: Interaction occurring in human colon. (Chassard *et al*., 2013)

Diseases and Dysbiosis

As there are approximately 10^{14} bacterial cells in the adult human gut, representing over 1000 different bacterial species. Among them, Bacteroidetes and Firmicutes are the two most common groups of bacteria found in the normal intestinal microflora.

In healthy people, there is cross-talk and cross-regulation between the host and the gut microbiota, resulting in a homeostatic balance of bacteria that keeps the gastrointestinal tract healthy and free of potentially pathogenic bacteria. The microbiota and the host have a

commensal relationship; the bacteria thrive in the gut's rich environment, while the host benefits from the bacteria's multiple functions.

The gut microflora's homeostatic balance is immensely advantageous to the host.

On the other hand, gut microbial alterations result in drastic imbalance between beneficial and potentially pathogenic bacteria, and makes the gut more vulnerable to pathogenic insult. This alteration in the microbial homeostasis is known as ‘dysbiosis’ which can be due to microflora imbalance, change in their functional composition and metabolic activities, or shifts in their local distribution. Generally, dysbiosis is classified into three types: (1) disappearance of beneficial microorganisms, (2) accelerated growth of potentially dangerous microorganisms, and (3) decline in overall diversity of microorganisms. These three types have been discovered to be non-exclusive and can occur at the same time, which is most often the case. (DeGruttola *et al*., 2016)

There is a lot of clinical and experimental evidence suggesting dysbiosis of the gut microflora plays a role in the development of various intestinal and extra-intestinal disorders. Intestinal disorders like inflammatory bowel disease, irritable bowel syndrome (IBS), coeliac disease and extra-intestinal disorders like allergy, asthma, metabolic syndrome, cardiovascular disease, and obesity. (DeGruttola *et al*., 2016; Carding *et al*., 2015)

Gastrointestinal Disorders

Inflammatory Bowel Disease (IBD): Reduced bacterial diversity is one of the characteristics of IBD patients' compositional alterations. As a result of the growth of ostensibly aggressive groups like Proteobacteria, Fusobacterium species, and Ruminococcus gnavus and in combination with reductions in protective groups like (such as Lachnospiraceae, Bifidobacterium species, Roseburia, and Sutterella)

Three pathogens are majorly linked with this disease, *Mycobacterium avium paratuberculosis, Escherichia coli* and *Clostridium difficile* which shows rise in the diseased person. Even so, there are no studies supporting a direct link between the rise in the number of these organisms and IBD. (DeGruttola *et al*., 2016; Tan *et al*., 2020; Bochenek *et al*., 2017)

Crohn’s disease and ulcerative colitis (UC) are the most common types of inflammatory bowel illness (IBD), Although the cause of both diseases is uncertain, growing evidence

suggests that gut microbial dysbiosis has a role in the development of IBD. (Carding *et al*., 2015; Kim *et al*., 2019)

In particular, *Clostridium difficile* (CD) shows rise in *Ruminococcus gnavus*, and a decline in *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, *Dialister invisus*, and an unknown of Clostridium cluster XIVa. With alteration in intestinal microbiota, an increase in mucin degradation and epithelial permeability has been observed. (DeGruttola *et al*., 2016; Hsu *et al*., 2019)

Extra-Gastrointestinal Disorders

Obesity: Obesity appears to be linked to a change in the ratio of Bacteroidetes to Firmicutes, which demonstrates a rise in the number of Firmicutes and decline in number of Bacteroidetes. In people with higher body weight and accumulation of body fat indicates higher disproportion in ratio of these organisms. (DeGruttola *et al*., 2016; Guirro *et al*., 2019)

Autism Spectrum Disorders: A disorder characterized by deficits in behavior and communication. Though, the cause of ASD is still unknown but there is a significant possibility that intestinal dysbiosis is involved in the development of ASD. The metabolites of the gut microbiota and the gut microbiota itself, appears to influence the central nervous system via the gut–brain axis. (DeGruttola *et al*., 2016; Li *et al*., 2021)

Cancer: The dysbiosis of the intestine is also linked with colorectal cancer (CRC). The risk factors associated with CRC are IBD, diabetes, obesity, as these diseases are linked with the dysbiosis of the intestine. Studies have shown drop in *Proteobacteria*, *Bifidobacteria*, *Prevotella*, and lower SCFA production rates, however there is a rise in *Firmicutes*, *Bacteroidetes*, *Enterobacteriaceae*, and *Fusobacteria*. Changes in the gut microbiota during dysbiosis have been linked to cancer and appear to play a significant role in this process. (DeGruttola *et al*., 2016; Chen *et al*., 2020)

Microflora Transplantation/ Recovery

The goal of microflora transplantation is to normalize intestinal dysfunction and repair boundary weaknesses in order to treat dysbiosis and recover microbiomes. These aims might be fulfilled using some old methods like as pro, prebiotics, drugs and devising new like nowadays fecal microbiota transplant (FMT); defined microbes in a synthetic mixture, possibly

adapted to a person's unique microbiome profile; drugs with high selectivity against major harmful bacterial species as well as modified intake. (Sartor *et al*. ,2016)

Probiotics

Probiotics are substances that contain viable organisms that help the host in the revival of the intestinal microflora. This is used when an imbalance of microflora occurs in the gut. They are generally safe but there are many disadvantages as it cannot be given to the patients with low immunity, in some cases it was found that harmful metabolites were produced. Apart from this it helps in the removal of pathogens and prevents the gut from being colonized by harmful microorganisms. (Blaut *et al*., 2002)

Probiotics are substances that inhibit the harmful activities of members of a microbial community. They can be used directly where there is infection to treat the infection and shut out the growth of the pathogens. (Olmos *et al*., 2001)

The use of probiotic is wide and can be used in the disease where there is a alteration in the gut flora or invasion of pathogens some diseases that are treated using probiotic are treat major diseases like inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), diarrhea, and gastrointestinal disorders, and recently have developed for new treatments like obesity, insulin resistance syndrome, T2D, chronic kidney disease, allergic asthma, prevention of dental caries. (Olmos *et al.,* 2001)

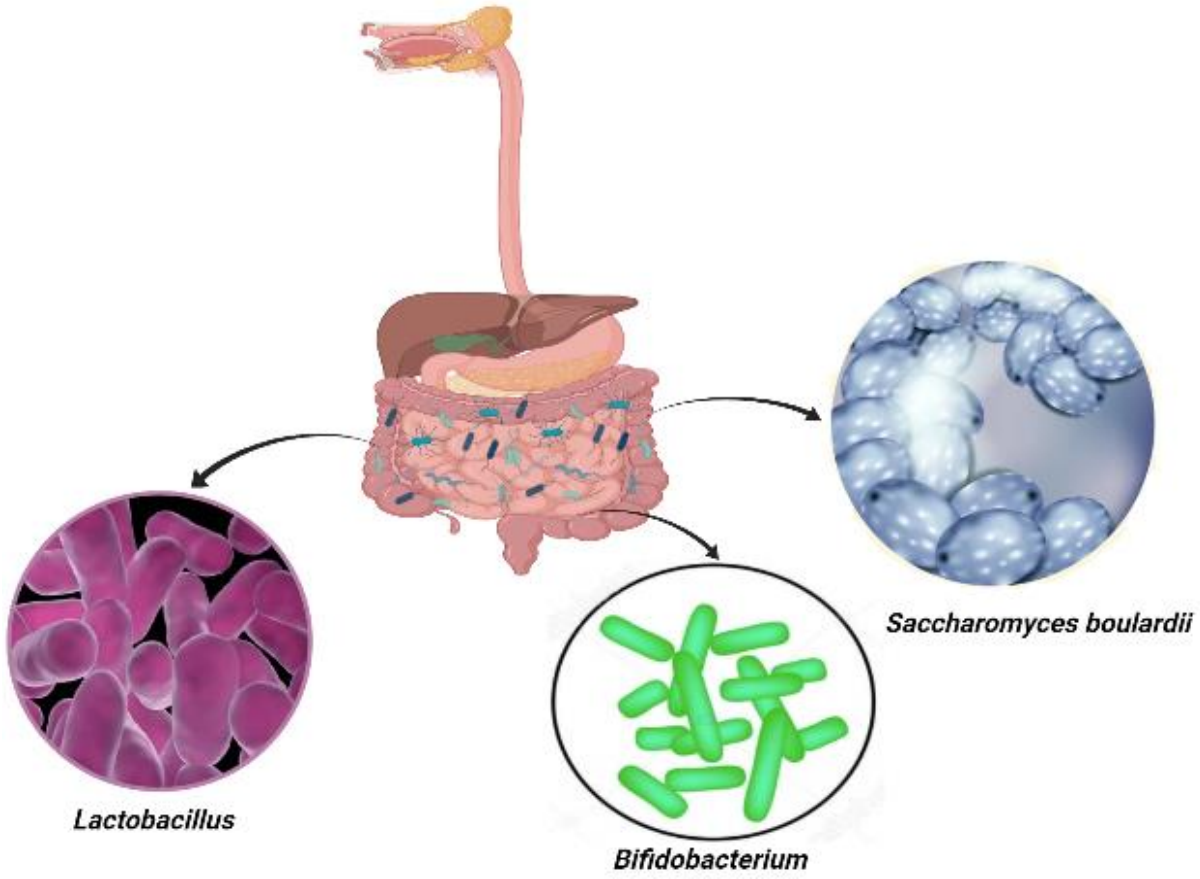

Figure 3: Probiotics are a combination of bacteria and yeast. *Lactobacillus* and *bifidobacterium* are two types of probiotic bacteria. *Saccharomyces boulardii* is the yeast most commonly found in probiotics (Created with BioRender.com).

Lactobacillus spp. Bifidobacterium spp. and *Saccharomyces boulardii* are frequently used as probiotics. (Collins *et al*., 1999). *Lactobacillus spp*. significantly decrease glucose, triglycerides, free fatty acids, and HbA1c in diabetic rats fed high fructose diets. (Yadav *et al*., 2008). Probiotic supplements containing *Bifidobacterium* and *Lactobacillus* improved glucose tolerance and the body cells are sensitive to response to insulin in women with diet-controlled diabetes during pregnancy. (Kim *et al*., 2019) The effective organisms are listed in Table 1.

Table 1: Probiotics beneficial effects

Probiotic	**Effects**	**Reference**
Lactobacillus acidophilus	Insulin sensitivity was higher. Insulin resistance is reduced.	(Asemi *et al*., 2013)
Lactobacillus casei	Improved insulin resistance and blood glucose levels.	(Qu *et al*., 2018)
Lactobacillus rhamnosus	Improves gastrointestinal function, oxidative stress, and inflammation in T2D.	(Segers *et al.*, 2019)
Lactobacillus reuteri	Reduces duration of rotavirus-induced diarrhea.	(Hsieh et al., 2013)
Bifidobacterium lactis	Increases imbalance of CD4+, CD25+, T lymphocytes, and natural killer cells in blood.	(Bengmark *et al.*, 2002)
Bifidobacterium bifidum	Protects against rotavirus-induced diarrhea.	(Blaut et al., 2002)
Bifidobacterium breve	Reduces symptoms of irritable bowel disease.	(Chin *et al.*, 2000)
Saccharomyces boulardii	Reduction of relapse in Crohn's disease patients in individuals with recurrent C. difficile-associated diarrhea, it lowers the risk of relapse.	(Jass *et al*., 2003)

Prebiotics

A prebiotic is a fermented component that alters the composition and activity of the microflora in the gastrointestinal tract (GIT) to benefit the host. Prebiotics can help the gut microbiota grow stronger and healthier as a key body organ. Fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose, polydextrose, and inulin are some examples of prebiotics. Other dietary fibers, such as larch arabinogalactan, resistant starch, beta-glucans, and xylooligosaccharides, are also included in the prebiotics (XOS). Beneficial effects of *Bifidobacteria* in the host include improved ingestion, increased mineral consumption, and immune strengthening. (Davari *et al*., 2019).

A vital role in the treatment of metabolic diseases, with significant implications for gut microbiota. Improvements in insulin sensitivity and a reduction in autoimmune responses are also possible outcomes. (Manzoor *et al.,* 2019).

Microbiota composition and functions are influenced by the presence of gut microbiota in which *Actinobacteria, Bacteroidetes, and Firmicutes* are the bacteria that can ferment FOS, GOS, and xylooligosaccharides. Fermentation of starch and fructans by *Bifidobacterium sp*. (Davari *et al*., 2019). Oat bran increases butyrate production, which is equivalent to FOS, as well as propionate production, which is higher than FOS, as well as *Bifidobacteria and Lactobacilli.* (Kedia *et al*., 2009) Inulin can only be fermented by a few butyrogenic bacteria due to its longer chain length, whereas FOS, which has a shorter chain length, can be fermented by a larger number of species. (Scott *et al.,* 2009)

Prebiotics improved hypercholesterolemia by lowering cholesterol absorption and encouraging microbial synthesis of SCFAs. (Kim *et al*., 2005). Prebiotics can help maintain glucose homeostasis, improve insulin resistance, and slow the progression of T2DM by altering the gut microbiome. (Wang *et* al., 2021) Other prebiotics including oligodextrin, lactose, starch that resist digestion, lactoferrin, were also reported to have the low presence of cholesterol level in T2D patients at risk to develop heart problems. (Gibson *et al*., 2004)

Antibiotics

Antibiotics are used to modify the flora, not to remove it. In the gut, many different types of bacteria reside, each with its own antibiotic susceptibility. (Quigley *et al.,* 2005) Antibiotics must be formulated in such a way that they target the pathogenic microbe while also affecting

some number of the host microbiome, that leave an impact on the intestinal microbiota even after the antibiotics have been removed or reduced. (Jernberg *et al*., 2010)

Several researches have examined the impact of antibiotics on microflora during diarrhea and inflammatory bowel disease. In high fat diet fed mice, administration of VSL#3 reduces hepatic natural killer T cells (the T cells that consider both natural killer (NK) receptors and T cell receptors and regulate inflammation), which also decrease inflammation by reducing NFkB activity in the liver and improves insulin sensitivity. (Yadav *et al*., 2008) By increasing insulin binding capacity to the liver membrane, oxytetracycline almost completely restored insulin sensitivity. Chronic oxytetracycline treatment significantly insulin response in spontaneously diabetic rats. (Bourassa *et al*., 1974)

A probiotic combination of *Bifidobacterium lactis, Lactobacillus acidophilus,* and *Lactobacillus rhamnosus* prevents alloxan-induced diabetes and improves sulfonylurea drug action in diabetics. (Membrez *et al.,* 2008) It has been reported that broad-spectrum antibiotic treatment, which included gut microflora modulation, improved glucose tolerance in mice. Change in food into energy such as acetate, a SCFA are well known to affect the metabolism and immune function of the host. (Cully *et al*., 2019) If antibiotics are required, assured interference may benefit to recover the gut microbiota to the normal stage. (Elinav *et al*., 2013) It has been discovered that certain probiotics hampered the microbiome while autologous fecal transplantation helped restore it.

Fecal Microbiota Transplantation

What is FMT?

Fecal microbiota transplant (FMT) is a new treatment strategy for improving the diseased person's altered gut microflora. (Wang *et al*., 2018) The fecal suspension, which is naturally rich in microorganisms and their metabolites and is derived from healthy donors and administered to the intestinal tract of an unhealthy recipient. (Borody *et al*., 2013) It will modify the altered gut microbiota by providing a microbiome with structural and functional balance from a suitable donor.

A variety of factors, including donors, recipients, and experimental environments, impact the survival of the microbiome in feces and its successful transfer. (Bokoliya *et al*., 2021)

For what it is used?

The goal of FMT is to improve gut dysbiosis by transporting stool from a healthy donor or well-defined colonic bacterial strains that include a stable, living, diversified, and typical microbial community. With the exception of fecal microbiota transplantation, a number of medical treatment approaches are now applied to treat gut imbalance, although many of them fail to generate good clinical results. FMT has been shown in several studies to be a beneficial therapy for a number of illnesses. (Wang *et al*., 2018)

Which diseases can be cured?

In the United States, FMT is known as a drug. There FMT is classified as a controlled substance. Currently, under the FDA's enforcement discretion policy, FMT can be used to treat clostridium difficile infection (CDI) and also useful for treatment of other conditions such as colon diseases, constipation, diabetes, overweight, nervous system damage, autism, several organ dysfunctions in censorious patients. (Wang *et al*., 2018; Bokoliya *et al*., 2021)

The majority of FMT's current clinical experience comes from treating recurrent or refractory clostridium difficile infections (CDI). FMT has been reported to be successful in order to frequent and obstinate clostridium difficile in numerous trials that might be because of the long-term recovery of the normal microflora after FMT. The treatment rate for recurring CDI is upto 90%, which is much greater than the 20-30% success rate of prolonged antibiotic therapy. As it is now mentioned the standard therapy for CDI recurring in various expert guidelines, and is the one sign recognized by the FDA (Food and Drug Administration) of the United States. (Wang *et al*., 2018) Since its efficacy in treating clostridium difficile infection, FMT has attracted a lot of interest as a viable treatment for a variety of additional gastrointestinal along with extra-gastrointestinal diseases. (Bokoiya *et al*., 2021) FMT can be used to treat inflammatory bowel disease (IBD), a medical illness characterized by abdominal discomfort or pain, as well as gut dysbiosis without a medical problem. Peptic ulcer and psoriatic arthritis act as the most prevalent immune-mediated disorders. (Patil *et al*., 2021)

The hope of changing gut flora is a chance as a treatment for overweight and raven syndrome has been investigated in many research. (Wang *et al*., 2018) FMT has also been shown to have therapeutic effects for peoples suffering from autism, microorganism resistant to a variety of drugs, and multiple organ damage in critical patients in a variety of case reports and animal

models. Furthermore, recent research to use an animal model and a severe study have shown that therapy for skin cancers with FMT is also effective. (Bokoliya *et al*., 2021)

Type 2 diabetes & dysbiosis

Type 2 diabetes

One of the main metabolic diseases around the world is Type II Diabetes Mellitus (T2DM), that is also called as hyperglycemia that occurs when insulin is produced by the beta cells of pancreatic but is not absorbed into the cell or the cell is not able to utilize the produced insulin and due to this the glucose present is the body is not absorbed. This condition is not only due to genetic factors but also due to lifestyle changes. Around the globe every 3rd individual is suffering from this condition, the identification of which is very tough not only that the cure of which is very costly and lifetime medication with strict eating habits is also necessary. This condition has a severe impact on nearby organs like heart, liver, kidney and due to this in these organs ill effects are seen. (Kharroubi *et al*., 2015; Yang *et al*., 2021)

The insulin hormone is very important and it also helps in the breakdown of various complex matter in our diet like carbohydrates, lipids and proteins these complex matter are broken down and as a result simpler material are produced and these release energy as they are simple to absorb by the cells, when these components are broken down by the hormone target tissues gets activated and function accordingly. But when there is a case of insulin resistance the target tissue does not function properly and in turn cause the abnormalities in the primary targets. The importance of insulin as an anabolic hormone causes metabolic abnormalities in carbohydrates, lipids, and proteins. These metabolic abnormalities are caused by low levels of insulin to achieve adequate response and/or insulin resistance of target tissues, primarily skeletal muscles, adipose tissue, and to a lesser extent, liver, at the level of insulin receptors, signal transduction system, and/or effectors, enzymes or genes. (Kharroubi *et. al*., 2015)

The chances of metabolic problem in people with T2DM was very high and they also had swelling in certain organs other than this it was also found recently that these people have a altered gut microbiome that means the normal state of the gut microorganism is changed and this condition is called as ‘gut dysbiosis’. (Yang *et al*., 2021)

The main role of the gut microbiota is that the aid in digestion, helps in the absorption of nutrients from the food, they act as 1st line of defense and prevents the colonization of pathogenic bacteria, they also help in maintaining the proper homeostasis of the individual in which they are residing. (Yang *et al*., 2021)

Link between type 2 diabetes and dysbiosis

It has been reported that gut microorganism plays an important role in T2DM there are chances of inflammation which can be related to obesity and other illness it was also reported that chances of T2DM increases when people tend to be more obese and this condition normally alters the gut microbiota the change can be deleterious to the host. Mainly the ratio between *firmicutes* to *bacteriodes* changes. (Yang *et al*., 2021; Everard *et al.*, 2013) This increase in intestinal permeability is caused by a number of factors that can be change in the composition of gut microbiota, change in the expression of the junction and can also be due to exceeding stimulation of the CB1 receptor.

It was found that humans in which gut imbalance occurs the constitution of the gut flora also changes where the beneficial bacteria are decreased in number and the which are responsible for microbiome alteration can be seen in high amount, the gram-positive bacteria aid in the many helpful process like the breakdown of bile acid that will be hindered when there is a imbalance in the microflora. From the previous studies it was also found out that in obese mice models (the mice were induced obesity with high fat diet) the level of gram-negative bacteria like *firmicutes* were high and gram-positive bacteria like *bacteriodes* were low (Everard *et al*., 2013), when this breach occurs the intestinal lumen becomes permeable to harmful bacteria as well as toxin from bacteria to enter and further cause complications. (Everard *et al*., 2013)

Effects of T2M

T2DM is caused not only by external factors but sometimes can also be caused by internal changes. This condition tends to have a damaged glucose transporter and other complications.

It was also reported that in this disorder the amount of proinflammatory cytokines is high and here the toxic substance can enter the circulatory system through the gut vascular barrier. (Yang *et al*., 2021)

In animal models where they were given a diet with high amounts of fat the alteration in the gut microbiome was seen as the result the intestinal immunity was compromised and the outer layer of both gram positive and gram-negative bacteria entered the bloodstream this in turn activated other immune cells leading to inflammatory state. (Yang *et al*., 2021)

In short, the amount of all proinflammatory compounds were high when a gut dysbiosis occurred.

Is there no cure for this condition?

The most common treatment strategy for T2DM is the administration of the drug Metformin. This drug has many advantages such as it helps us to reduce the body weight, has a neutral effect and is a cardio protective, it has also been noted that the drug is more effective when administered orally rather than intravenously, about 30 percent of drug is eliminated from the

body is hence is most widely used for the treatment of T2DM but it was also noted that harmful effect of the drug metformin that is it tends to alter the gut microbiota and thereby increasing the LPS producing bacteria and also this in turn produce SCFA. (Elbere *et al*., 2018)

What can be done to avoid the above disadvantage?

It was found that by infusing intestinal microorganism from a thin healthy donor to a obese mice the insulin resistance was decreased and the dysbiosis was restored (Everard *et al*., 2013) the above process is called Fecal Microbiota Transplantation.

This is one of the most promising techniques that was used to cure many diseases in which the gut alteration was found. The fecal to be transferred must be taken from healthy donor which has to be screened properly to avoid further complication and see if the donor is healthy or not the fecal matter from the screened healthy donor will be viable and as processed that can be transplanted into the diseased organism (Chanyi *et al*., 2017; Bokoliya *et al*., 2021)

Reports suggested that the use of FMT was seen to cure many diseases in which the gut dysbiosis occured like one of the diseases where the injection is caused by the *Clostridium difficile*, other disease can be related to bowel disorder. (Wang et al., 2021)

Why do we expect it to work in a scenario?

Effect of FMT on low blood sugar in type 2 diabetes was studied by reducing glucose intolerance and regenerating damaged cells, resulting in some kind of prospective type 2 diabetes therapy method. (Wang *et al*., 2019) Although further research on the broader effects of gut bacteria is still in its early stages, but if gut health does affect overall health, fecal transplants could be the one to be used as treatment. When previous therapies fail, this novel approach may help to restore gut health naturally, allowing good bacteria to proliferate, combat illness and enhance a person's overall health. (Sethi *et al*., 2019)

HYPOTHESIS

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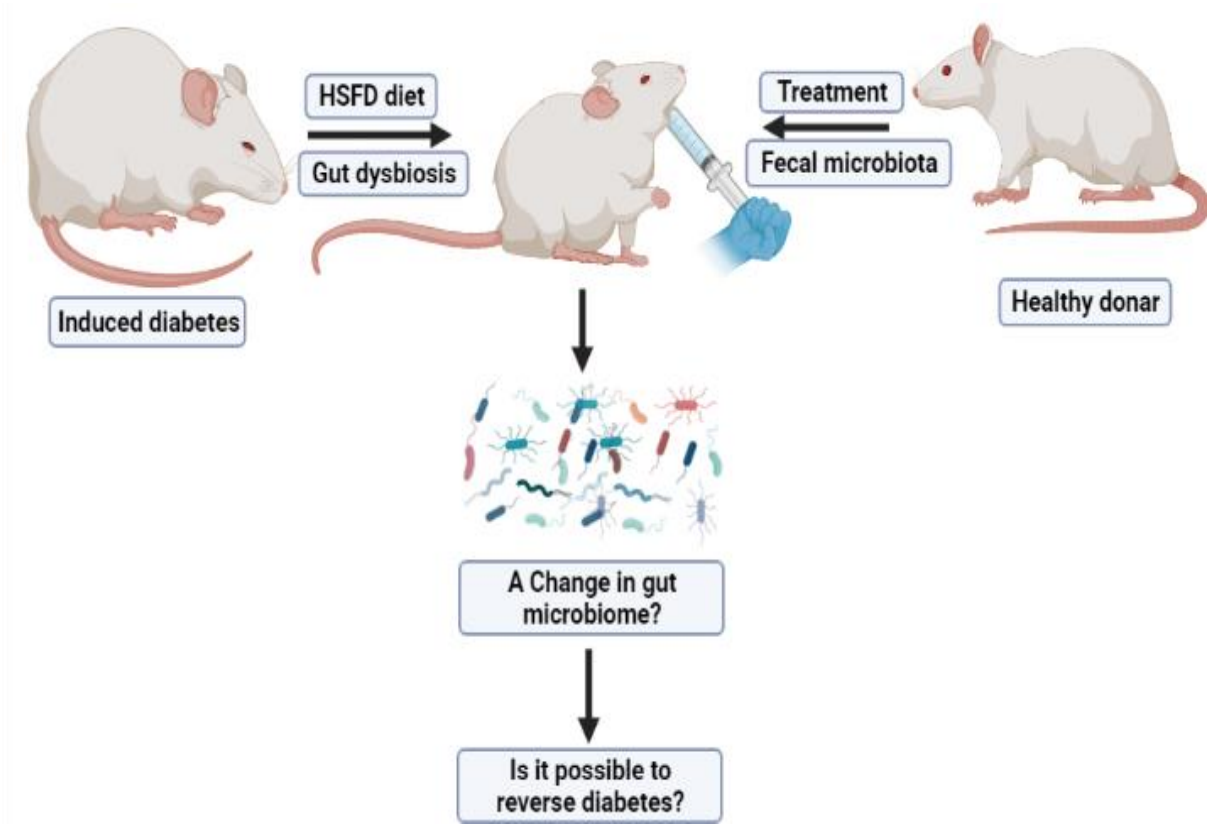
Dysbiosis occurs when the ratio of microflora is disturbed. High sugar fat diets can cause alteration in the microflora which can lead to diabetes, metabolic disorders and other related diseases as there is a huge role of microflora in maintaining a homeostasis. The present study aims to evaluate the reversal potential of fecal microbiota transplantation (FMT) for the treatment of diet induced diabetes.

Fecal microbiota transplantation (FMT) is a type of super probiotic. Probiotics are the 'good' bacteria that have a positive impact on our health. In FMT, the 'good' bacteria come from the fecal of a healthy donor which is then transplanted into the recipient's gastrointestinal tract in order to restore the gut alteration.

So, we hypothesize that the administration of fecal microbiota transplantation (FMT) in T2DM might be useful and effective for its treatment.



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OBJECTIVES

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1. Identification and characterization of healthy donors.
2. Evaluating the reversal potential.

MATERIALS & METHODS

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Animals and Ethical Approval:

Male, Wistar rats, weighing 250-300 g, were obtained from Zydus Pharmaceuticals and housed at Central Animal Facility, Institute of Pharmacy, Nirma University, Ahmedabad. After the acclimatization for 2 weeks, the rats were subjected to the experiment. The rats were kept in plastic cages with RO water and normal diet (chow pellets). The animal laboratory conditions were under control with proper temperature and humidity. Later, the rats were fed with their respective water and diet according to the grouping as mentioned in the table (Table no. 3). The animal research had been carried out according to the moral suggestions for the care and use of laboratory animals of the Institutional Animal Care and Use Committee, Nirma University, Ahmedabad; under the CPCSEA guidelines of Ministry of Environment and Forest, New Delhi (protocol No. IS/FAC/2020/029). And the rats were randomly divided into different groups as mentioned below. (Table 2)

Table 2: Experimental groups and their treatments

Serial No.	Groups	No. of Animals	Treatment
1	Normal Control (NC)	3	-
2	Diabetic Control (DC)	3	-
3	DC + Standard drug	3	Metformin (100 mg/kg of body weight)
4	DC + FMT	3	Processed Fecal

Type 2 diabetes was induced to the DC, DC + Standard drug and DC + FMT group with the help of a high sucrose fat diet for 3 (12 weeks) combined with 4% fructose water, 3 consecutive days and 1 day RO water and 65% sucrose dosing. At different intervals, OGTT was measured to check the induction of diabetes. After induction, Rats in the DC + FMT group were orally administered with processed fecal sample for 3 months (12 weeks); Rats in the DC + Standard drug group were orally administered with metformin (40 mg/kg) daily for 75 days; NC rats and DC rats were monitored throughout the study without any intervention. The OGTTs levels were observed during the experiment in all groups. Every 2- or 3-days stool sample was collected and stored/frozen at -80 °C. And the body weight was taken twice a week.

Table 3: Diet Compositions for Normal and HSFD Rats

Diet Compositions (%)	Normal Diet	High Sucrose Fat Diet
Corn oil	5	5
Choline chloride	0.2	0.2
Casein	20	20
D-Methionine	0.3	0.3
Dalda	0	30
Fructose	0	17.5
Sucrose	0	30
Starch	65	0
Salt mix	3.5	3.5
Vitamin mix	1	1
Wheat bran	5	5

Treatment Strategy:

Fecal Microbiota Transplantation (FMT):

The selection of the donor animal was done on the basis of its physical appearance, normal body mass index, biochemical test and screening of stool samples. The fecal pellets of that

donor animal were collected in an empty 1.5 ml tube using a sterile forcep. The tube was quickly closed and kept in the ice box. Later, it was kept at -80°C for storage until use.

Fecal sample processing:

Fecal sample (100 mg) was dissolved in 2 ml PBS (Phosphate Buffer Saline), mixed well and centrifuged for use. This is for 1 rat, so in the multiplication of 3 the things were taken. So, 300mg of fecal sample was dissolved in 6 ml PBS. Then, vortexed and centrifuged 300-500 rpm, for 6 mins at RT (room temperature). The supernatant obtained is the processed fecal sample used for the treatment. The administration was done using a cannula attached to a syringe. The treatment was scheduled as mentioned in Table 4. And the treatment strategy used for the same was firstly removing the diet and administering omeprazole (20 mg/kg) for the preparation of the gut. (Segawa *et al*., 1987). And then 3 hours later giving the FMT (100 mg/rat) treatment. (Bokoliva *et al*., 2021)

Table 4: Treatment Schedule for FMT

No. Of Week	Treatment
Week 1	4 continuous days
Week 2	Alternate days
Week 3	4 continuous days
Week 4	Alternate days
Week 5	4 continuous days
Week 6	Alternate days
Week 7	4 continuous days
Week 8	Alternate days
Week 9	4 continuous days
Week 10	Alternate days
Week 11	4 continuous days
Week 12	3 days before autopsy

Oral Glucose Tolerance Test:

To check if the rats were diabetic or not OGTT was performed at different intervals in the induction phase. For that the diet was removed 12 hours prior to the test. From the test fasting blood glucose level was estimated and then dextrose (glucose) solution was administered orally to the rats after 0 min reading according to their body weight (2gm/kg) and the estimation was done by making a small cut on the tail end, and from the blood measuring the glucose level with the help of glucose strips and free style optium H blood glucose monitor (Abbott, UK) at 0 min, 30 min, 60 min and 120 min. Graph was plotted with the help of GraphPad Prism 5 software and Area under the curve for glucose ($AUC_{glucose}$) was determined using the trapezoidal rule. (Jena *et al*., 2014)

Autopsy Schedule:

Autopsy of Normal Control, Diabetic Control and Standard drug groups had been done at 76^{th} day while the autopsy of FMT group had been done at the end of the treatment phase i.e., at the end of 3 months (12 weeks). From the dissected rat small intestine, large intestine, liver, spleen, pancreas and adipose tissue were collected. The collected tissues were stored in the refrigerator at -20°C, to isolate the RNA from it. And small parts of the tissues were collected and outsourced for the formation of histology-slides. Blood and cecal samples were collected as well.

Serum Separation:

From the dissected rats, blood was collected with the help of a syringe by puncturing the heart. The blood was collected in two 1.5 ml eppendorf tubes from each rat and was kept at room temperature for 4-5 hours. Later, it was centrifuged at 3000 rpm for 20 mins at 4°C to obtain the serum. From the extracted serum different biochemical tests were performed, such as Glucose, HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), SGOT (Serum Glutamate Oxaloacetate Transaminase), SGPT (Serum Glutamate Pyruvate Transaminase) and TG (Triglycerides) with the help of protocol as provided by manufacturer of the Lab-care diagnostics kit which we used for the experiment.

Fecal Analysis:

Fecal samples were collected twice a week and were stored at -80°C. Also, colonic fecal (cecal) was collected during the autopsy. These samples were used for further microflora studies.

Colony forming Unit (CFU):

CFU count was performed by the serial dilution from fresh fecal sample and processed fecal sample to check the bacterial load.

DNA Isolation from Fecal Sample:

DNA was isolated from the fecal sample with the help of a kit by the following protocol given by QIAamp® Fast DNA Stool MiniKit (cat no. 51604).

Microbial Quantification by RT-PCR:

The presence of DNA was checked with the help of 1.5% agarose gel electrophoresis and the quantification was done with the help of nanodrop and was further used for PCR using 16s rRNA gene specific primers.

Table 5: Forward and reverse primer sequence of the bacteria were used in the study with standardized Tm and amplicon size of the product.

Name	**5'-3' sequence**	**Temperature (℃)**
Bifidobacterium	F- 5'GCGTGCTTAACACATGCAAGTC 3' R- 5' CACCCGTTTCCAGGAGCTATT 3'	F:66.7℃ R:66.1℃
Escherichia coli	F- 5' CATGCCGCGTGTATGAA 3' R- 5' CGGGTAACGTCAATGAGC 3'	F:63.1℃ R:61.8℃

Short Chain Fatty Acid (SCFA) Analysis by HPLC:

High-performance liquid chromatography (HPLC) was performed from fecal samples of animals.100 mg of fecal was pooled and vortexed in 2ml of HPLC buffer and centrifuged at 10,000 rpm for 15 minutes at 4˚C. The supernatant was collected. The final volume was made up to 2ml. 10 µl of syringed filter sample was injected in HPLC for the analysis. 5 mg/ml acetate, propionate and butyrate standard were prepared in the same buffer solution. Samples were run in the agilent column for 15min.

Histopathological Analysis:

A small portion of the small intestine, large intestine, liver and adipose tissues were removed from the tissues of the dissected rats from each group during the autopsy and the fixation of tissue was done using 10% formaldehyde. The tissues were sealed in a coating of paraffin wax. And were outsourced for the formation of histology-slides. Where, a 5µm section was sectioned using a microtome, and slides were made and stained with Hematoxylin and Eosin (H&E) stains which was observed under a microscope. (Prajapati *et al*., 2016)

Gene Expression Analysis:

RNA Isolation:

RNA was isolated from the tissue of the animal. 100mg of tissue was put to the homogenizer tube and then homogenized with 1000µl of RNA isoplus. The homogenate was then transferred to an eppendorf tube and stored on ice for 5 minutes before centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant was transferred to a 1.5 ml eppendorf tube and 200µL of chilled chloroform was added. It was vortexed gently (a milky solution was formed) for 5 minutes at room temperature. After that, the sample was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant (100-200µl) was transferred into a fresh tube. The chilled isopropanol, of around 0.5-1.0x the volume of the upper layer collected, was added to the fresh tube and gently stirred. The tubes were then kept on ice for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 100µl of ethanol. Samples were centrifuged at 8,000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet was air dried to let ethanol evaporate. The pellet dissolved with 150µl of nuclear free water and then loaded on to gel and the concentration was checked in Nanodrop and the gel was used for cDNA synthesis and gene expression studies.

Primer designing for gene expression studies

Integrated DNA technologies were used to design primers for the various genes. Primers for various genes were designed, as shown in Table 6. The primer sequence was blasted against the nucleotide sequence of each gene to ensure that the primers were completely aligned with the mRNA sequences of the respective genes.

Table 6: Details of primers used for gene expression studies

Gene	**5'-3' sequence**	**Temperature (℃)**
TLR-2	F: TGCAGAGCAACGATGGAGAAA R: ACAGCAGCGTCAGGGTGAAG	F: 68.1℃ R: 67.9℃
TLR-4	F: GGCTGTGGAGACAAAAATGACTC R: AGGCTTGGGCTTGAATGGAGTC	F:68.7℃ R:69.5℃
NFkB	F: CCCCACGAGCTTGTAGGAAAG R: CCAGGTTCTGGAAACTGTGGAT	F:66.8℃ R:66.2℃

Statistical Analysis

Results were presented as mean ± SE, mean ± SEM. A statistical difference between the means of the various groups was analyzed using one way analysis of variance (ANOVA). A statistical difference between the means of the various groups was analyzed using two-way analysis of variance (ANOVA) The statistical significance was then evaluated by using Graphpad prism software (V 5.01).

RESULTS

RESULTS

Colony Forming Unit (CFU):

CFU count was performed from the fresh fecal and processed fecal to check the bacterial load present in the fecal. The bacterial load was 2.9 x 10^6 of the fresh fecal and 2.8 x 10^6 of the processed fresh fecal. So, the results obtained were almost the same in both samples.

Physiological Parameters:

Bodyweight

The diabetic control (DC) animal group has shown weight gain as compared to the normal control group. In the case of reversal groups FMT and Metformin, the group has shown a decrease in body weight compared to the DC group but there is no significance. Thus it is an indication that modulation of microflora by FMT has a positive effect in terms of weight gain. (Figure. 4-A) With respect to the body weight, the required change in the organ weight has been observed. (Figure. 4-B)

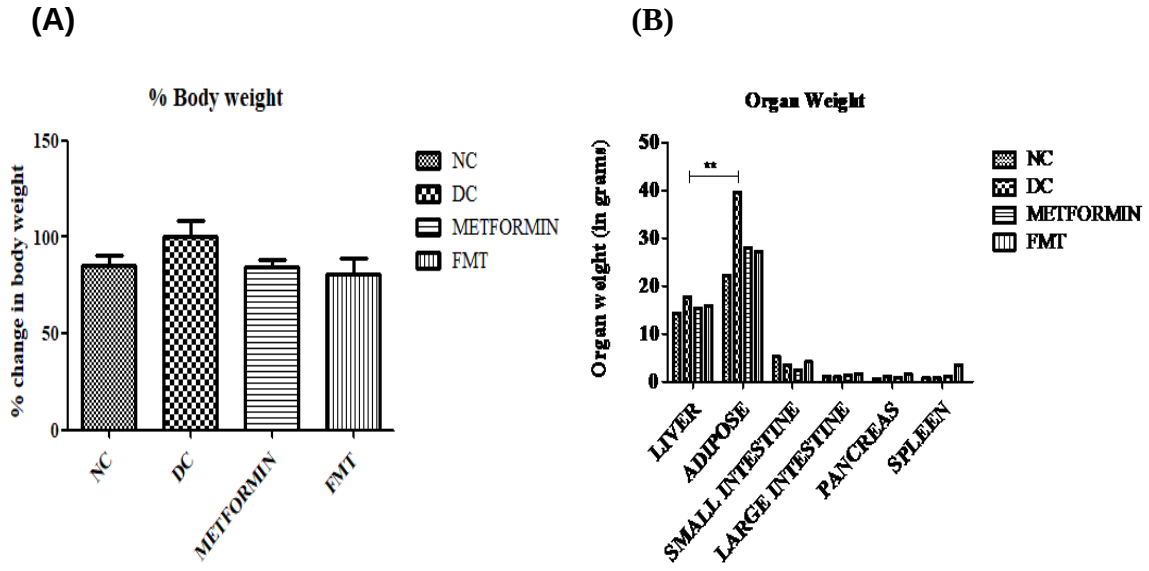

Figure 4: Physiological Parameters. (A) Body Weight (B) Organ Weight. Data are presented as mean ± SEM calculated as each group. Two-way ANOVA was performed for organ weight. **Represent comparison with control at the same point. GraphPad Prism v5.01 used for statistical analysis, Significance is indicated as **p<0.01.

Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance test was performed to check glucose utilization after oral administration of glucose to overnight fasted animals. All the animals were given glucose (2ml/kg) orally and the levels of glucose were checked at 0, 30, 60 and 120 minutes of glucose administration. After glucose administration, rise in glucose levels were observed in all animals after 30 minutes which was completely utilized after 120 minutes in a control group of animals. In case of normal control rats, the level of glucose shot immediately after 30 min of glucose dose but gradually decreased within 120 min and came to a normal level. The level of glucose in diabetic control rats drastically shot up and could not come back to normal range. The level of glucose in metformin rats shot immediately after 30 min and gradually decreased within 120 min and came to a normal level. The level of glucose in FMT rats shot immediately after 30 min and gradually decreased within 120 min and came to a normal level. (Figure. 5-A) Area under curve of glucose, level at the normal range after 120 min of glucose feeding. Normal control showed significant level of glucose tolerance ***($p < 0.005$) compared to Diabetic control. Normal control showed significant level of glucose tolerance **($p < 0.05$) compared to Metformin and FMT while Diabetic control showed significant level of glucose tolerance ###($p < 0.005$) compared to Metformin and FMT. (Figure. 5-B)

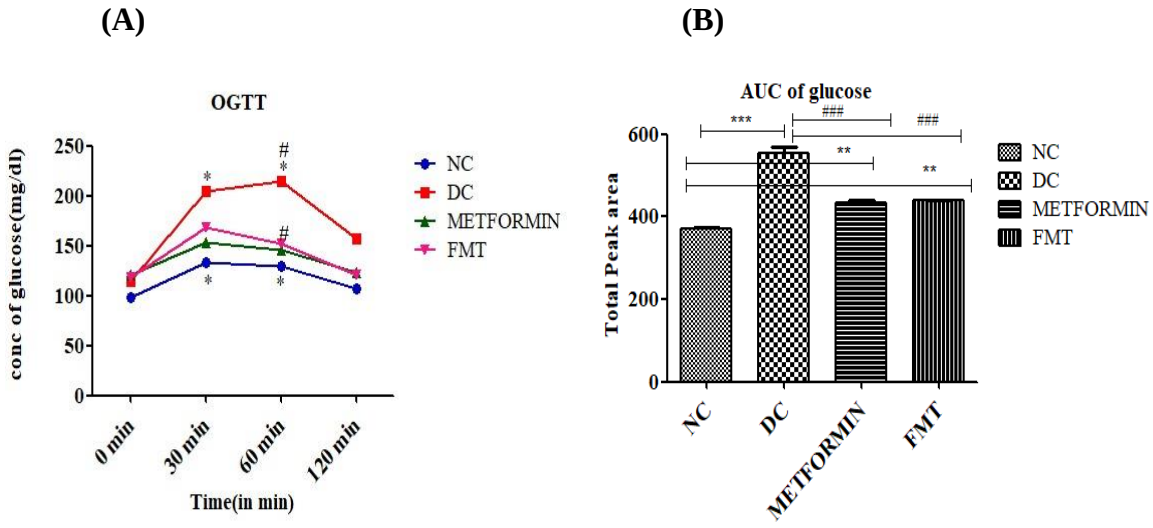

Figure 5: Oral Glucose Tolerance Test. (A) OGTT: Rats were fasted overnight before OGTT. Blood glucose levels were measured every 30 min for 120 min after oral glucose dose, * Represent comparison with normal control and # comparison with diabetic control. (B) Relative areas under curves (AUC) for OGTT. Data are presented as mean ± SEM calculated as each group; two-way ANOVA performed for OGTT and one way ANOVA for AUC

GraphPad Prism v5.01 used for statistical analysis, Significance is indicated as ***p<0.005, **p<0.05, *p <0.05, #p<0.05 and ###p<0.005.

Serum Biochemical Profiling

Serum biochemical analysis of glucose, (TG) Triglycerides, HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), SGOT (Serum Glutamate Oxaloacetate Transaminase) and SGPT (Serum Glutamate Pyruvate Transaminase) tests were performed, with the help of protocol as provided by manufacturer of the diagnostic kit (Accucare reagent kit by Lab-care diagnostics). Metformin and FMT groups had shown reversal to some extent but due to a smaller number of animals and variations in individual animals the results were not significant. (Table 7)

Table 7: Serum Biochemical Profiling of each group

Serum Biochemical	NC	DC	Metformin	FMT
Glucose (mg/dl)	155.28 ± 43.38	316.68 ± 69.33	306.25 ± 136.22	177.82 ± 22.78
Triglycerides (mg/dl)	86.83 ±15.14	270.25 ± 25.83	130.58 ± 24.29	165.19 ± 85.28
HDL (mg/dl)	19.24 ± 3.68	31.25 ± 4.49	12.73 ± 1.74	25.93 ± 2.24
LDL (mg/dl)	36.72 ± 6.53	71.10 ± 9.51	40.10 ± 3.03	46.98 ± 19.94
SGPT (U/L)	33.32 ± 12.69	20.97±13.67	37.16 ± 16.56	39.37 ± 9.60
SGOT (U/L)	109.36 ± 10.03	81.77 ± 2.09	143.08 ± 35.56	147.77 ± 36.6

SCFA Analysis by HPLC:

The excreted fecal samples were taken from the animals before they were dissected. And prior to the injection of samples in HPLC, all samples were processed with HPLC buffer, centrifuged, and filtered. Samples were analyzed using a standard sample peak, retention duration, and peak area. Bar graphs represent the molar concentrations of acetate, propionate, and butyrate.

The primary SCFA metabolites of gut flora are acetate, propionate, and butyrate. The presence of SCFA in the stomach indicates bacterial activity. The level of SCFA in feces can be

determined using the HPLC technique. Butyrate and Propionate are anti-inflammatory, whereas acetate is pro-inflammatory.

As seen below Acetate is higher than butyrate and propionate. (Figure 6 A, B & C) As of acetate shoot because of pro inflammatory characteristics but in order to compensate the inflammation, butyrate and propionate also showed a significant elevation in this study in which it is showing a reversal in Metformin and FMT groups. (Figure 6 A, B & C)

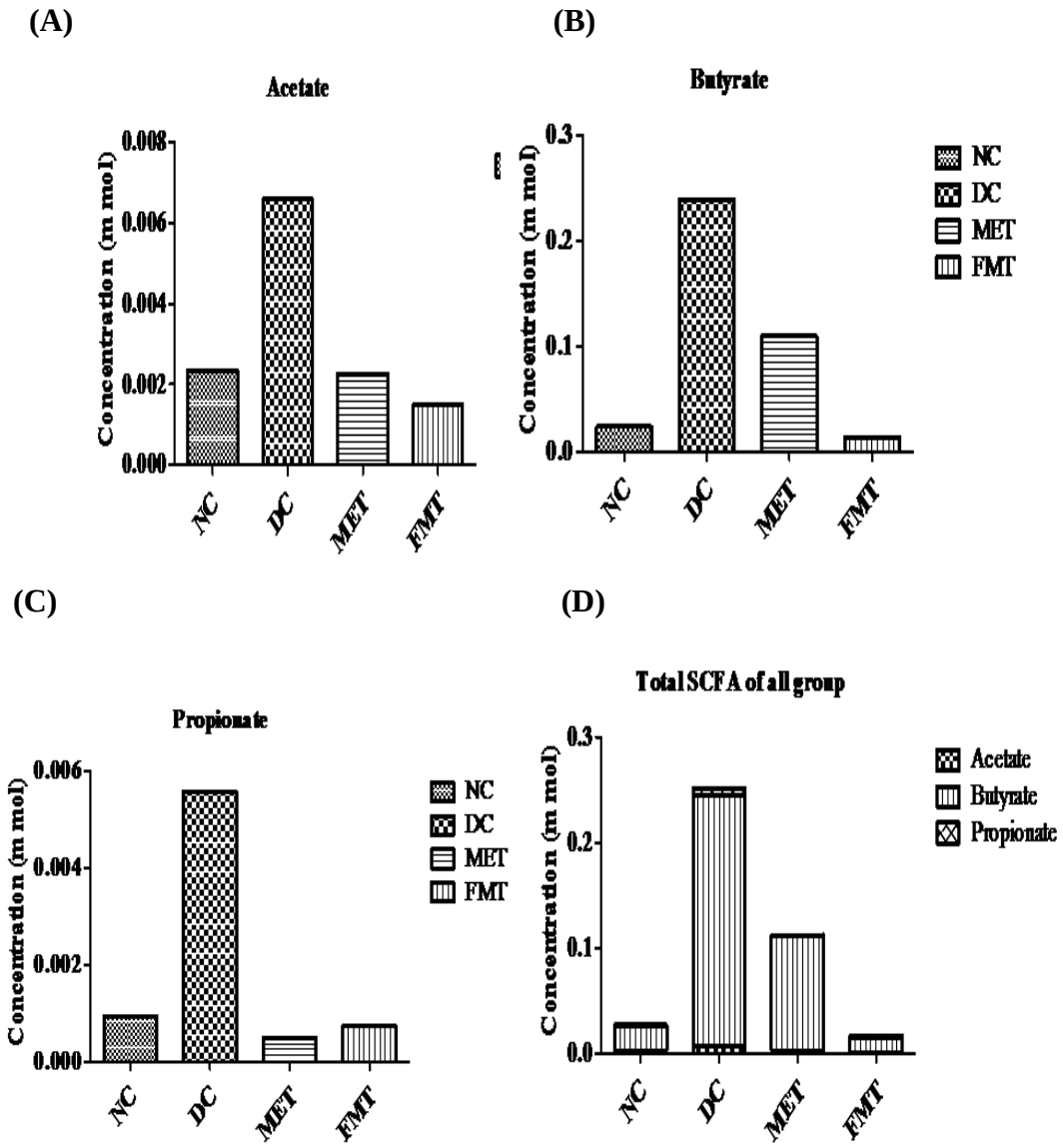

Figure 6: HPLC of SCFAs from fecal matter. Acetate, propionate and Butyrate were measured and concentration of SCFAs was calculated based on standard solution. Data is represented as SCFA concentration in millimolar. Values are represented as mean ± SEM as each four group.

Histopathological studies:

Hematoxylin-Eosin (H&E) staining is the most extensively used staining method to study tissue histology. Eosin stained the cytoplasm pink, while hematoxylin of the H&E stain stained the nucleus purple. It can clearly show the cytoplasm, nucleus, and extracellular matrix. (Fischer *et al*., 2008) This staining can be used to study tissue structure, cellular structure, and tissue injury.

The Small intestine is the portion of the gastrointestinal (GI) tract. Normal morphology was observed in the small intestine of normal control group (Figure 7-A) and structure of crypt cells was distorted in diabetic control group (Figure 7-B) and they show recovery in reversal groups, Metformin and FMT. (Figure 7- C, D)

(A) **(B)** **(C)** **(D)**

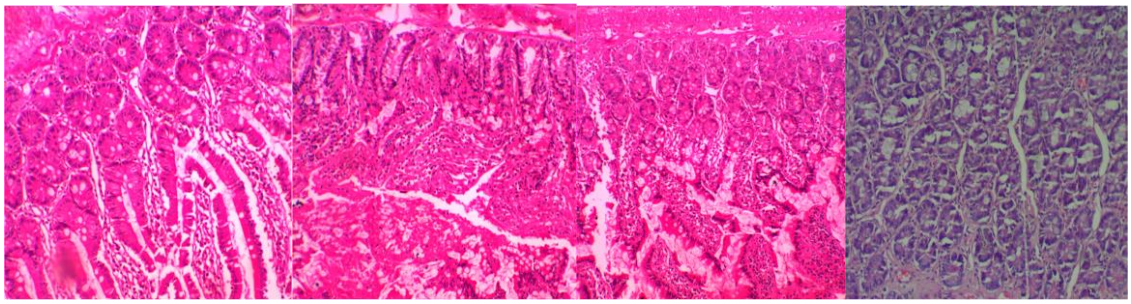

Figure 7: Histopathological analysis of SI. Hematoxylin and eosin-stained cross sections of SI region of (A) Normal Control, (B) Diabetic Control, (C) Metformin (D) FMT. Image captured using Abcam software and all are at 100x magnification. Crypt cells.

The large intestines of the diabetic control group were found to be distorted (Figure 8-B) compared to normal morphology of the normal control group (Figure 8-A) and the recovery was observed in both Metformin and FMT groups. (Figure 8-C, D)

(A) **(B)** **(C)** **(D)**

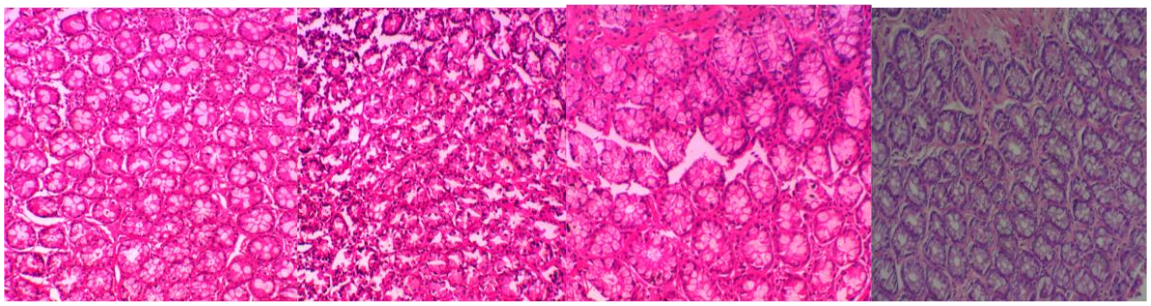

Figure 8: Histopathological analysis of LI. Hematoxylin and eosin-stained cross sections of the LI region of (A) Normal Control, (B) Diabetic Control, (C) Metformin, (D) FMT. Image captured using Abcam software and all are at 100x magnification.

The liver of diabetic control group (Figure 9-B) showed inflammation inside the central vein compared to normal morphology of the normal control group (Figure 9-A) and the recovery was observed in both Metformin and FMT groups. (Figure 9-C, D)

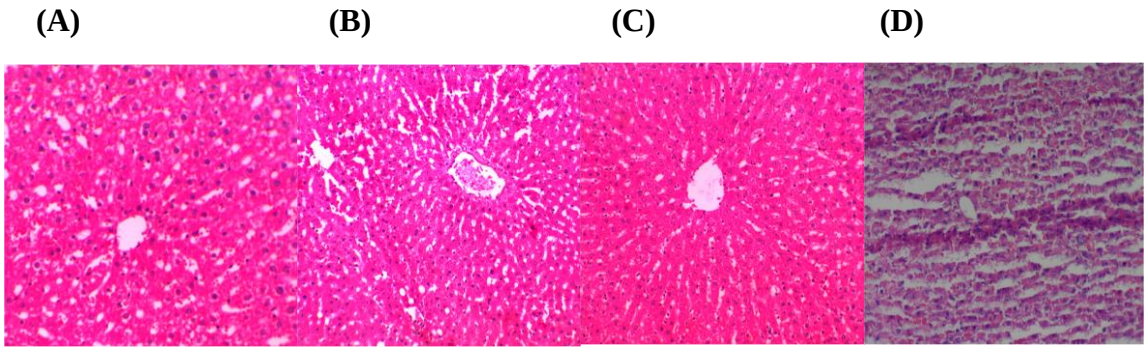

Figure 9: Histopathological analysis of Liver. Hematoxylin and eosin-stained cross sections of Liver region of (A) Normal Control, (B) Diabetic Control, (C) Metformin (D) FMT. Image captured using Abcam software and all are at 100x magnification. CV- Central vein.

A notable difference was seen in the size of adipose cells when comparing to normal control group (Fig 10.A) to diabetic control group (Fig 10-B) and they show recovery in both Metformin as well as FMT group (Fig 10 C, D)

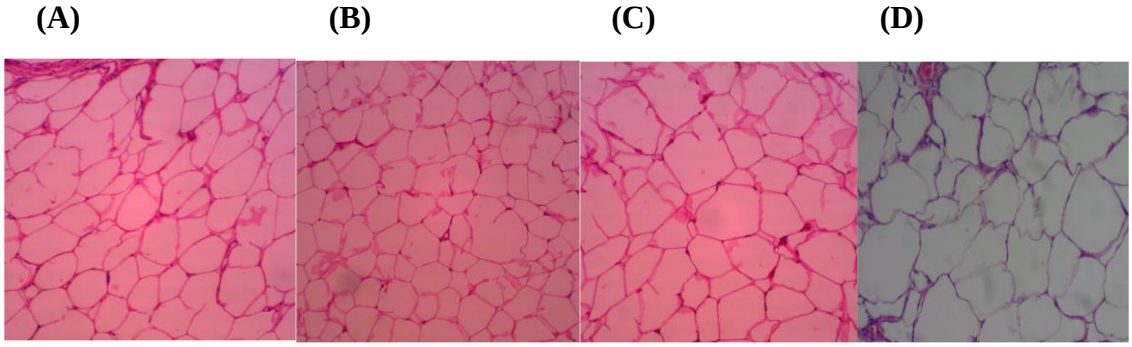

Figure 10: Histopathological analysis of Adipose. Hematoxylin and eosin-stained cross sections of Adipose region of (a) Control, (b) Diabetic Control, (c) Metformin, (d) FMT. Image captured using Abcam software and all are at 100x magnification. FC- Fat cells.

Densitometric analysis:

16s rRNA gene sequence of E.coli and Bifidobacterium was performed with the help of specific primers to characterize the microbiota present in the fecal by RT-PCR, and were checked using densitometry technique. The result obtained was of normal control, diabetic control and metformin group in which FMT result was not found. A normal control group showed significant abundance of E. coli**(p <0.01) compared to diabetic control group while diabetic control showed significant abundance of e. coli ##(p <0.001) compared to the metformin group. A normal control group showed significant abundance of *Bifidobacterium**(p <0.05) compared to diabetic control group while diabetic control showed significant abundance of *Bifidobacterium* #(p <0.05) compared to the metformin group.

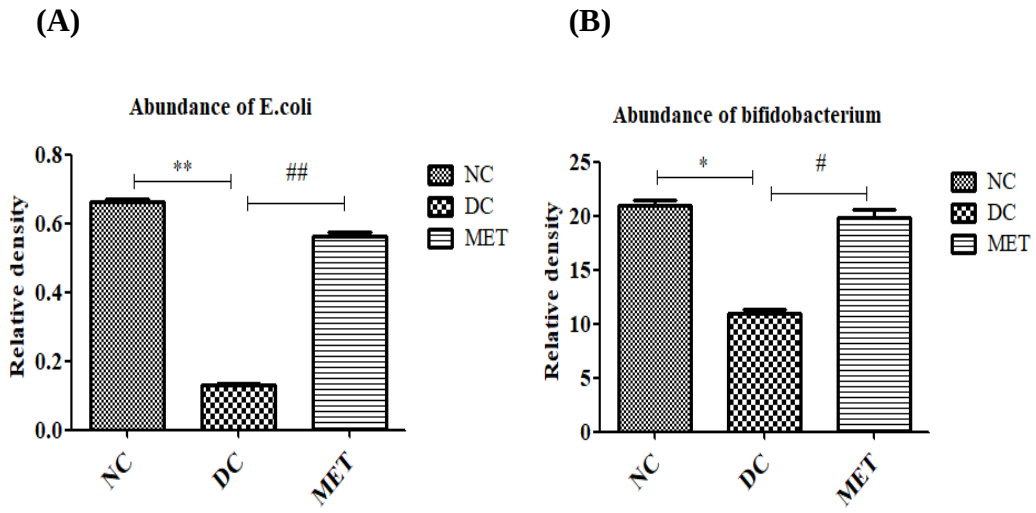

Figure 11: Densitometric Graph of NC, DC, Metformin and FMT. (A) Abundance of E.coli (B) Abundance of Bifidobacterium. Data is presented as mean ± SEM calculated as each group; Two-way ANOVA was performed to calculate the abundance of e-coli and abundance of *Bifidobacterium*. GraphPad Prism v5.01 used for statistical analysis, Significance is indicated as **p<0.01, *p <0.05, #p<0.05 and ##p<0.01.

Gene Expression Studies:

Gene Expression studies were carried out for the liver tissues. TLR-4, TLR-2, NF-kB.The result not obtained as the reason might be the primers used were old or because the theoretically optimum temperature is too high or low for the annealing of primers.

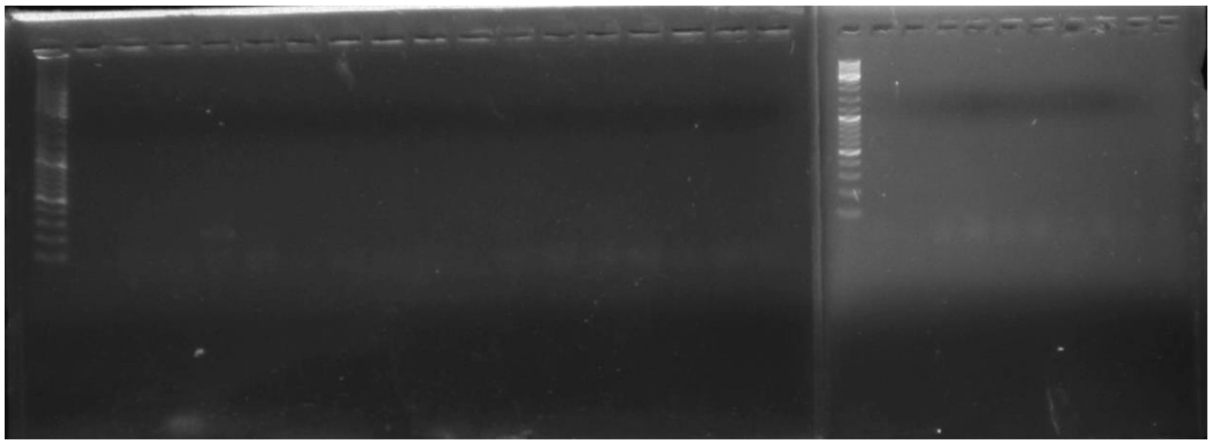

Figure 12: Expression study of TLR -2, TLR-4 and NF kB. Lane1: Ladder, lanes 2-7: TLR-2, lanes 8-13: NF-KB and in next lanes 14- 19 of gel for TLR-4.

DISSCUSSION

DISCUSSION

The microorganisms present in the human body plays an important role in digestion, providing immunity and also helps in the synthesis of many vitamins. The gut contains gram positive and gram negative microbes in equal ratio and any change in this ratio is referred to as gut dysbiosis; to cure this condition many treatment options are available like the prebiotic, probiotic and the use of antibiotics.

The consequence of using antibiotics was that the microorganisms were becoming resistant against them. In order to restore this dysbiosis recently used process is the transplantation of fecal matter from healthy donors, and the success rate of FMT was clearly visible in curing the CD infection. (Oksi *et al*., 2013)

T2DM is a condition where insulin produced by the beta cells of the pancreas, the cells are not able to utilize the produced insulin and due to this the glucose present in the body is not absorbed which leads to the accumulation of glucose in the blood. (Kharroubi *et al*., 2015)

High sucrose high fat diet was given to animal models (rats) that mimicked the T2DM and it was found that due to this, the body weight of rats was increased (Malone *et al*., 2019) that was visible morphologically. The diabetes induced by the HSFD diet in the rats was confirmed by performing OGTT, a glucose intolerance was seen supporting the fact that they are diabetic, these diabetic rats had glucose intolerance.

As a part of reversal, FM (Zhang *et al*., 2020) was used from the healthy donor, the healthy donor was also of the same strain and same species, the fecal sample was collected and stored in -80 °C and at the time of dosing the fecal sample was taken and processed by dissolving in PBS and administered to rats for a duration 12 weeks. To compare the results of the FMT group, another group with standard metformin treatment was kept.

Although an increase of body weight is observed in the diabetic control as they were fed with a high sugar fat diet, it has been reported that the high sugar fat diet leads to gain in body weight (Rasool *et al*., 2018) and the body weight was reduced using FM, which has been reported; after the FM treatment, the body weight decreases. (Yong *et al*., 2013)

After the treatment phase the reversal was checked using OGTT, which showed that in comparison to the diabetic group, both the reversal groups had a significant decrease in the

glucose level. That is coming somewhat near to the normal. (Wang *et al*., 2020) This indicates that after the FM treatment in the diabetic model, reversal is observed.

And to check the other parameters for the reversal study, an autopsy was scheduled after 12 weeks of treatment. Organs and blood were collected and weight was taken for further use for the histopathological, gene expression & serum biochemical analysis, respectively.

With respect to the body weight, the appropriate change has been observed in the organ weight. As diabetic control's adipose tissue weight was high compared to other groups. (Chait *et al*., 2020) This indicates that due to a high sugar fat diet, which promotes the T2DM, adipose tissue level will increase.

In serum biochemical analysis the result showed that the metformin and FMT groups showed some sort of reversal compared to the DC groups but the result was not significance (Tizhe *et al*., 2013) had reported that one of the reasons being non-significant could be due to the low number of experimental animals and also the variation which may be due to differences in individual animals.

The primary SCFA metabolites of gut flora are acetate, propionate, and butyrate. So, the level of SCFA in feces was determined using the HPLC technique, where shoot up in the acetate was observed which indicated the proinflammatory characteristics in the diabetic control group, where as in reversal groups, metformin and FMT to compensate that the butyrate and propionate level showed a significant elevation. Butyrate and propionate indicate the anti-inflammatory characteristics, which indirectly indicates the reversal. (Riviere *et al*., 2016) it has been reported the abundance of butyrate and propionate will decrease by the time.

The histology study of the small intestine, large intestine and liver was done in which, normal analogy was observed in the normal control group and distorted structures was observed in the diabetic control group. The further signs of recovery were shown in the reversal groups, metformin and FMT. (Nambiranjan *et al*., 2018; Hu *et al*., 2022; Okada *et al*., 2007) And it has been reported that histopathology of the SI, LI and liver cells of the T2DM model is swollen and distorted whereas in the metformin and the FMT recovered normal cells could be observed. The histological analysis of adipose did not show the desired results but in comparison to controls bit of recovery is observed. The importance of adipose cell size in health and disease is reported in paper. (Stenkula *et al*., 2018)

As 16s rRNA gene sequence of E. coli and Bifidobacterium was performed with the help of specific primers to characterize the microbiota present in the fecal by RT-PCR, and were checked using densitometry analysis technique. The result obtained was of normal control, diabetic control and metformin group; FMT group results were not found. A normal control group showed significant abundance of E. coli and *Bifidobacterium* against the diabetic control group and diabetic control showed significant decrease in abundance of *Bifidobacterium* compared to the normal group. So, the dysbiosis caused by the downregulation of the gram-positive bacteria, *Bifidobacterium* indirectly indicates an increase in gram negative bacteria which occurs in the T2DM dysbiosis. So, if we would be able to successfully treat the reversal groups the equilibrium in the number of gram negative and positive organisms would be observed. (Zhu *et al.*,2020) As reported that the imbalance of microbes in dysbiosis occurs and with the balance of microbes the reversal is seen.

And the gene expression study was carried out from the RNA isolated from liver tissues using TLR-4, TLR-2, NF-kB primers. The result was not obtained, as the reason might be that the primers used were old or because the theoretically optimum temperature is too high or low for the annealing of primers compared to the practical temperature. But if the positive results were obtained by the FM treatment in reversal groups (FMT and metformin) then TLR-2 would have increased as compared to the increase in TLR-4 in diabetic group.

CONCLUSION

CONCLUSION

In our study we are inducing diet induced diabetes into male wistar strain rats by feeding them with HSFD diet for a induction period of 3 months and at the end of this period it was found that the body weight, blood glucose level and cholesterol were high: this confirmed T2DM in the positive control group of rats and this was compared with the normal control group.

For the FMT treatment fecal sample was collected from the healthy male wistar strain rat and stored in -80 °C, before the treatment the fecal sample was processed using PBS and was administered to the diabetic rat; the treatment phase was also of 3 months and at the end of 3 months, OGTT was performed along with other biochemical tests to check the reversal potential of FMT.

The results showed the signs of reversal, the body weight of rats decreased; along with that the comparison of all the groups showed favorable results in serum biochemical profiling, histological data, SCFA by HPLC, gene expression when compared to the Metformin group.

The immediate future aspects of FMT would be increased animal numbers for experimental studies, the carbohydrate, inflammatory and cytokines pathways can be elaborated and studied. The effects of SCFA receptors can be identified. It can also be formulated for targeted delivery in the GI Tract. The long-term goal of FMT could be the clinical trials extended in multiple animal models to check the efficacy of FMT and using FM as a personalized medicine.

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