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Metagenomics of Gut Microbiota in Diabetic Animal Model Treated with Neeradimuthuvallathy Mezhugu - A Herbomineral Siddha Formulation

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Abstract: Diabetes, a chronic metabolic disease marked by high blood glucose levels, has become an epidemic globally. Despite their effectiveness in many situations, current treatment methods have drawbacks, such as the establishment of medication-resistant diabetes, which is commonly accompanied by insulin resistance, negative side effects, and the development of drug resistance. 'Siddha', one of the ancient Indian Systems of Medicine, has many drugs in use that treat diabetes effectively. This study examined the effects of Neeradimuthuvallathy Mezhugu (NM), a Herbomineral Siddha formulation, on the gut flora of diabetic rats. NM had a significant antidiabetic activity in the animals and also a more notable beneficial effect on the gut microbiota than the common anti-diabetic medication glibenclamide. Rats with diabetes showed decreased microbial diversity and an altered bacterial composition, with more potentially hazardous bacteria and fewer helpful bacteria. Beneficial bacteria including *Lactobacillus* and *Bacillus* were enriched; microbial diversity was greatly increased, while the number of harmful bacteria was decreased after NM therapy. With the high NM dosage, Group 6 (HD) animals showed the most noticeable results, with the most diversity and enrichment of good bacteria such as *Bacillus celluloses* and *Lactobacillus acidophilus*. These results imply that NM could be beneficial for the treatment of Diabetes by altering the gut flora in these patients. Furthermoreclinical trial research is required to validate these findings and explore the full therapeutic potential of NM in the treatment of diabetes.

Keywords: Gut Microbiota, Antidiabetic, Neeradimuthuvallathy Mezhugu, Siddha Formulation, Metagenomics.

1. Introduction

Diabetes

Globally, diabetes mellitus, a metabolic disease marked by hyperglycemia, has become an epidemic (Forman et al., 2017). Cardiovascular disease, neuropathy, nephropathy, and retinopathy are among the complications that can result from abnormalities in insulin secretion, insulin action, or both (American Diabetes Association, 2023). High blood pressure and hyperlipidaemia linked to diabetes form a cluster

of diseases that are extremely difficult to treat with a single medication. This forces medical practitioners to treat diabetes with numerous pharmacological therapies, which ultimately results in negative side effects for diabetic patients (Alberti, 2005; Deedwania & Volkova, 2005). As a result, it is increasingly critical to seek out drugs that are both effective at treating diabetes-related disorders and free of adverse reactions.

A range of pharmacological strategies is used in allopathic medicine to treat diabetes, with a primary emphasis on controlling blood glucose levels. For instance, drugs such as sulfonylureas (glipizide, for example) increase the pancreatic output of insulin (Inzucchi et al., 2019), while metformin improves peripheral tissue insulin sensitivity (Nathan et al., 2009). Acarbose and other alpha-glucosidase inhibitors decrease the absorption of glucose by slowing down the intestinal breakdown of carbohydrates (Chiasson et al., 2003). Dapagliflozin and other SGLT2 inhibitors encourage the kidneys to excrete glucose (Ferrannini, 2019). Moreover, drugs such as DPP-4 inhibitors and GLP-1 receptor agonists increase insulin production while inhibiting glucagon release (Drucker & Nauck, 2006). Insulin therapy, delivered by injection or pump, becomes crucial when oral drugs are not enough (American Diabetes Association, 2023).

Gut Microbiota

The gut microbiome, including bacteria, fungi, viruses, and archaea, is a dynamic and complex microbial ecology in the human gut (Human Microbiome Project Consortium, 2012). According to Thursby and Juge (2017), this complex community is essential for several physiological processes, such as immunological regulation, nutritional metabolism, and gut barrier integrity maintenance. Dysbiosis, or imbalances in this ecosystem, has been linked to the aetiology of several illnesses, including diabetes (Qin et al., 2012).

Diabetes and Gut Microbiota

Research has repeatedly shown that people with diabetes have changes in the makeup of their gut microbiota. Among these alterations is a decrease in good bacteria like *Lactobacillus* and *Bifidobacterium*, which are recognised to be essential for preserving immunological and gut health. On the other hand, people with diabetes have often been found to have higher levels of potentially pathogenic bacteria, including *Bacteroides* and *Ruminococcus* (Karlsson et al., 2013; Qin et al., 2012).

Additionally, research has demonstrated that people with diabetes have a less diverse gut microbiota, which suggests a less resilient and robust microbial ecosystem (Le Chatelier et al., 2013). This diminished variety may impact numerous facets of host health, which may also compromise the gut microbiota's general function. In relevance to this, potential treatment options for metabolic disorders like diabetes are provided by the developing field of microbiome-based treatments (Ottman et al., 2017). Preclinical and clinical research have demonstrated encouraging outcomes when altering the gut microbiota with probiotics, prebiotics, and faecal microbiota transplantation (Carding et al., 2015).

'Siddha' System of Medicine

However, Diabetes has conventionally been treated by one of the Ancient Traditional Indian Medical Systems- 'Siddha' (Balasubramanian, 2013). Siddha Medicinal preparations are mostly a concoction of Polyherbal formulations that comprise medicinal plants or Herbomineral formulations that comprise medicinal plants and minerals. A study has reported that more than 800 medicinal plants have been shown to have hypoglycemic effects in ethnopharmacological evidence to date. Many of these plants are extremely noteworthy for their antidiabetic capabilities, making them a target for isolating antidiabetic molecules (Rizvi & Mishra, 2013). Hence, there is a wealth of scientific evidence supporting the use of these medicinal plants in Traditional medicinal systems like 'Siddha' to treat diabetes mellitus,

particularly when it comes to the isolation, characterisation, and targeting of specific bioactive chemicals that are specific to diabetes (Ezuruike & Prieto, 2014; Patel et al., 2012). By blocking the AMPK/PI3K/AKT signalling pathway's metabolism in muscle and pancreatic cells, the plant extracts of traditional 'Siddha' medicine and their individual bioactive metabolites have been shown to lower blood glucose levels (Tabatabaei-Malazy et al., 2015; Waltenberger et al., 2016; Costa et al., 2020; Francini et al., 2019; Mazibuko-Mbeje et al., 2018). It stresses a comprehensive strategy that includes certain dietary and lifestyle changes, mineral preparations, and herbal formulations (Kannan, 2011). As a result, it is obvious that the Siddha system of medicine has several beneficial phytocompounds that could potentially treat diabetes.

Given this, Neeradimuthuvallathy Mezhugu (NM) is one such Siddha formulation that has been used for hundreds of years to treat diabetes. A traditional Siddha formulation with a long history of use, Neeradimuthuvallathy Mezhugu (NM) has various indications including diabetes and cancer. The ingredients of Neeradimuthuvallathy Mezhugu (NM) are listed in Table 1 below. In addition, the Chinese herb Scutellaria baicalensis contains the phytocompound Baicalin, a crucial part of NM that has been shown to have anti-hyperglycemic effects (Simna et al., 2012). According to studies, the gut flora of diabetics can be altered by Chinese herbal formulas that contain Baicalin (Xu et al., 2016). Fortunately, the HPLC analysis of the phytochemical constituents of NM also illustrates Baicalin as an important phytochemical in the drug (Simna et al., 2012), indicating that it could be a potential anti-diabetic compound.

Table 1. Ingredients of Neeradimuthuvallathy Mezhugu (NM)

S.NO.	VERNACULAR NAME (TAMIL NAME)	BOTANICAL NAME
01	Serankottai	Semicarpus anacardium
02	Purified Neeradimuthu	Hydnocarpus kurzii
03	Parangipattai	Smilax china
04	Pirappan kizhangu	Calamus rotang
05	Karunseeragam	Nigella sativa
06	Seeragam	Cuminum cyminum
07	Vasambu	Acorus calamus
08	Sivanarvembu	Indigofera aspalathoides
09	Sanganver	Azima tetracantha
10	Karudan Kizhangu	Corallocarpus epigaeus
11	Amukkura Kizhangu	Withania somnifera
12	Vellarugu	Enicostemma littorale
13	Erukkanver	Calotropis gigantean
14	Athipattai	Ficus racemosa
15	Saranaiver	Boerhaavia diffusa
16	Milakaranai Ela	Toddalia asiatica
17	Veppam paruppu	Azadiracta indica
18	Vetpalai arishi	Wrightia tinctoria
19	Ellu	Sesamum indicum
20	Neeli samoolam	Indigofera tinctoria
21	Purified rasam	Hydragyrum

S.NO.	VERNACULAR NAME (TAMIL NAME)	BOTANICAL NAME
22	Purified Ganthagam	Elemental Sulfur
23	Purified thurusu	Copper Sulfate
24	Purified pal thuththam	Zinc Sulfate
25	Nei	Bos indicus

Given the link between diabetes and the gut microbiome and the possibility that NM could alter both, evidence reveals that Siddha herbal formulations containing many phytocompounds and the probiotic adjuvants used in these formulations may contribute to their positive impact on gut health by encouraging the growth of beneficial gut bacteria (Devaki et al., 2023).

The aim of the present study is to investigate the effect of NM on the gut microbiota in diabetic animal models. Given the known connection between diabetes and gut dysbiosis, as well as the possible impact of Siddha medicine on metabolic health, the purpose of this work is to use 16S rRNA sequencing to examine the effects of Siddha drug NM on the gut microbiota of diabetic rats. The objective of the present study is to analyse the gut microbiota in diabetic and drug-treated animals. The effective method for identifying and describing the bacterial populations in the gut microbiome is 16S rRNA gene sequencing. A highly conserved section of bacterial DNA, the 16S rRNA gene, comprises both conserved and variable regions. Researchers can categorise bacteria to the genus or species level by sequencing these variable areas (Caporaso et al., 2010). Without having to cultivate specific bacterial species, which can be difficult for many gut microbes, this method allows researchers to thoroughly examine the diversity and makeup of gut microbial communities (Schloss et al., 2009). Research on the microbiome has been transformed by 16S rRNA gene sequencing, which has shed light on the intricate relationships between human health and illness and the gut microbiota.

2. Materials and Methods

Animal Model and Diabetes Induction

Wistar albino rats weighing 180–200g and aged between two and three months were chosen for the study. Diabetes was induced with streptozotocin (55 mg/kg) intraperitoneally. After 72 hours of Streptozotocin injection, animals with blood glucose levels over 250mg/dl were considered diabetic and included in the study. The animals were grouped and included in the study for 28 days with appropriate drug treatments as mentioned in Table 2. The Standard Control used was Glibenclamide

Table 2. Experimental grouping of animals and treatment dosages

S. No.	Animal groups	Sample size	Dosage
1.	Group 1 – Vehicle Control	Male:3; Female: 3	Ghee
2.	Group 2- Diabetes Induced	Male:3; Female: 3	Streptozotocin (55mg/kg)
3.	Group 3- Positive Control	Male:3; Female: 3	Glibenclamide (5mg/kg b.wt.)
4.	Group 4- Diabetes Induced + NM (LD)	Male:3; Female: 3	Streptozotocin (55mg/kg) + NM (200 mg/kg b.wt.)
5.	Group 5- Diabetes Induced + NM (MD)	Male:3; Female: 3	Streptozotocin (55mg/kg) + NM (400mg/ kg b.wt.)
6.	Group 6- Diabetes Induced + NM (HD)	Male:3; Female: 3	Streptozotocin (55mg/kg) + NM (800mg/ kg b.wt.)

7.	Group 7- Diabetes Induced + NM (MD+Probiotics)	Male:3; Female: 3	Streptozotocin (55mg/kg) + NM (400mg/kg b.wt.) + Lactobacillus 10^8 CFU

Sample Collection and Genomic DNA Isolation

Once the study period was completed, samples of faeces were taken from 4 animals (2M + 2F) of every animal group and given the code for each sample as mentioned in Table 3. Using the QIAamp DNA Stool Mini Kit from Qiagen, microbial DNA was extracted from the faecal samples. Samples are placed in a bead-beating tube for quick and complete homogenization. Cell lysis occurs through mechanical and chemical methods. A total genomic DNA is captured on a silica membrane using a spin-column format. The DNA is subsequently washed and eluted from the membrane, making it ready for NGS, PCR, and other downstream applications.

Table 3. Animal Samples and Sample Code

S.NO.	SAMPLE NAME	SAMPLE CODE
01	GROUP-1;MALE-1	I-M1
02	GROUP-1;MALE-2	I-M2
03	GROUP-1;FEMALE-1	I-F1
04	GROUP-1;FEMALE-2	I-F2
05	GROUP-2;MALE-1	II-M1
06	GROUP-2;MALE-2	II-M2
07	GROUP-2;FEMALE-1	II-F1
08	GROUP-2;FEMALE-2	II-F2
09	GROUP-3;MALE-1	III-M1
10	GROUP-3;MALE-2	III-M2
11	GROUP-3;FEMALE-1	III-F1
12	GROUP-3;FEMALE-2	III-F2
13	GROUP-4;MALE-1	I-M1
14	GROUP-4;MALE-2	I-M2
15	GROUP-4;FEMALE-1	I-F1
16	GROUP-4;FEMALE-2	I-F2
17	GROUP-5;MALE-1	II-M1
18	GROUP-5;MALE-2	II-M2
19	GROUP-5;FEMALE-1	II-F1
20	GROUP-5;FEMALE-2	II-F2
21	GROUP-6;MALE-1	III-M1
22	GROUP-6;MALE-2	III-M2

23	GROUP-6;FEMALE-1	III-F1
24	GROUP-6;FEMALE-2	III-F2
25	GROUP-7;MALE-1	I-M1
26	GROUP-7;MALE-2	I-M2
27	GROUP-7;FEMALE-1	I-F1
28	GROUP-7;FEMALE-2	I-F2

Purification, PCR Amplification, and Library Preparation

Once the 16S rRNA gene was amplified using the V3-V4 primers, the amplified PCR products were purified using the QIAquick PCR Purification Kit. The quantity and quality of the PCR products were evaluated using a nano-spectrophotometer. The library was prepared by fragmenting genomic DNA. Following DNA fragmentation, adapters for Illumina sequencing were introduced, and the products were amplified using eight PCR cycles, providing a distinct index for every sample. Following library amplification, their concentration was assessed using the tape station. The Illumina NovaSeq 6000 platform was then used to perform 300 cycles of paired-end sequencing on the libraries that had been mixed at 0.7 nM ratios.

Clustering of the Sequenced Reads

Based on their unique barcodes, samples were assigned paired-end readings, which were subsequently eliminated along with the primer sequences. FLASH (V1.2.7) (Magoč & Salzberg, 2011), an exceptionally quick and accurate analytical tool, was utilised to combine paired-end reads when at least a portion of the reads overlapped with the read generated from the opposite end of the same DNA fragment; the splicing sequences were known as raw tags. Raw readings were subjected to quality filtering using the QIIME (V1.7.0) quality-controlled technique (Bokulich et al., 2013; Caporaso et al., 2010).

The tags were compared to the reference database SILVA using the UCHIME approach (Edgar et al., 2011) to detect chimaera sequences, which were then eliminated (Haas et al., 2011). This resulted in Effective Tags, which were then investigated further.

OTU Cluster and Taxonomic Annotation

The Uparse program (v7.0.1090) analysed the sequences using all of the effective tags (Edgar & Robert, 2013). Sequences that were part of the same OTUs were more than 97% identical. For each OTU, a sample sequence was selected for further annotation. QIIME (Version 1.7.0) (Altschul et al., 1990) in the Mothur method was performed for each representative sequence against the SSUrRNA database of the SILVA Database (Wang et al., 2007) in order to perform species annotation at each taxonomic rank (Quast et al., 2013). Further, the MUSCLE (Edgar, 2004) (Version 3.8.31) tool was used to determine each OTU's phylogenetic relationship. The abundance of OTUs was normalised using a sequence number standard that matched the sample with the fewest sequences.

To further investigate the phylogenetic relationships within the genus, an evolutionary tree was constructed using the aligned representative sequences of the top 100 genera. Figure 1. shows the phylogenetic tree for the 16S data at the genus level. The resulting normalised data was used for subsequent alpha diversity analysis. All of the indices (Chao1, Shannon, Simpson, and ACE) in our

samples were shown using R (Version 2.15.3), and their calculations were performed using QIIME (Version 1.7.0). Additionally, beta diversity was calculated on both weighted and unweighted unifrac to evaluate the differences in species complexity between samples using the QIIME tool (Version 1.9.1). A cluster tree was created using clustering technique to look at the similarities among the samples. The Unweighted Pair-group Method with Arithmetic Mean (UPGMA), an ecological hierarchical clustering technique, examines sample commonalities. To create a new node with a branching point half the distance from the initial two samples, samples that are closest in distance are clustered together. After calculating the average distance between the new node and the previous samples, the two closest samples are once more located to finish the clustering tree and merge all of the samples.

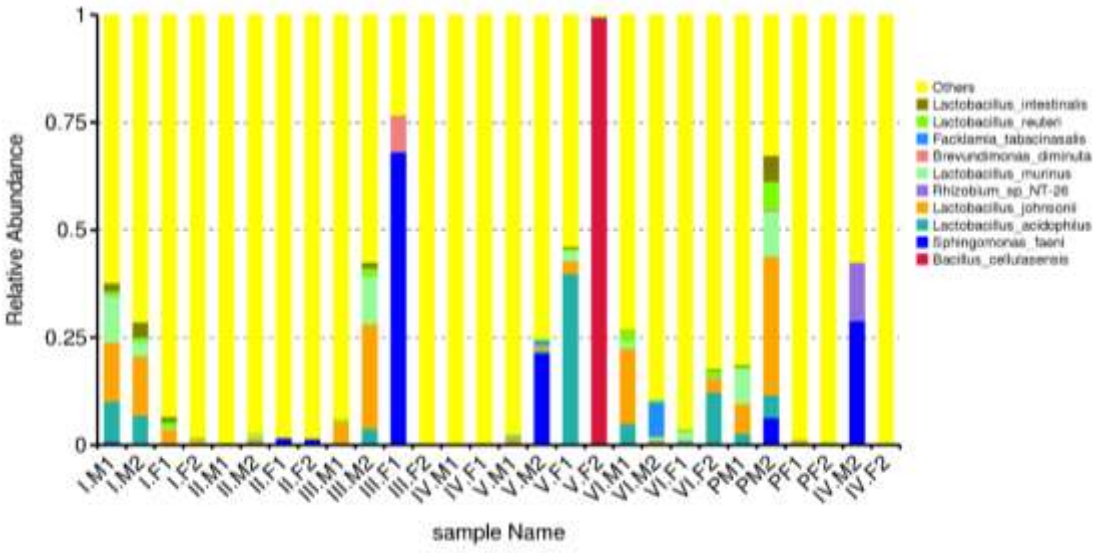

Fig.1 Taxa relative abundance at the species level

PcoA Analysis

Principal component analysis (PCA), which was used to lower the dimension of the original variables using the ade4 and ggplot2 packages in R software (Version 4.0.3), came before cluster analysis. To obtain the central coordinates and visualise complicated, multidimensional data, Principal Coordinate Analysis (PCoA) was used. A weighted or unweighted unifrac distance matrix between previously acquired samples was converted to a new set of orthogonal axes, where the first principal coordinate shows the maximum variation factor, the second main coordinate shows the second maximum one, and so on. The ade4 and ggplot2 packages in R software (Version 4.0.3) were used to display the PCoA analysis. Additionally, data dimensions were decreased by using non-metric multidimensional scaling (NMDS). Similar to the PCoA, NMDS makes use of a distance matrix, but its primary focus is numerical rank. Only the rank information, not the numerical differences, may be represented by the distance between sample points on the diagram. R software, namely the ade4 and ggplot2 packages, was used to perform the NMDS study.

3. Results

Microbial Community Analysis

Filtering and Classification of Reads

The NovaSeq 6000 platform (Illumina) was used to sequence all of the obtained genomic DNA. The

QIIME (V1.7.0) quality control approach was used to the sequenced reads, and the raw/clean reads from male animals in Group 1 (Control) were 204030/196667 (I M1) and 202041/194088 (I M2). 205498/198001 (II M1) and 204588/197394 (II M2) were found in Group 2 (Diabetic). 205498/198001 (III M1) and 204588/197394 (III M2) were found in Group 3 (Glibenclamide). Group 4 (NM (LD)) had IV M1 and IV M2 values of 203860/196522 and 201997/194426, respectively. NM (MD) Group 5 had V M1 of 206623/198417 and V M2 of 217860/209680. These were 202834/194900 (VI M2) and 206106/198529 (VI M1) for Group 6 (NM (HD)). 205610/197556 (PM1) were found in Group 7 (NM (MD) + Probiotics) and 208747/200430 (PM2) raw/clean reads. The raw/clean reads for female animals in Group 1 (Control) were 118416/115620 (I F1) and 213858/206260 (I F2). 118416/115620 (II F1) and 213858/206260 (II F2) were found in Group 2 (Diabetic). 118416/115620 (III F1) and 213858/206260 (III F2) were found in Group 3 (Glibenclamide). There were 167029/161124 (IV F2) and 215462/206721 (IV F1) in Group 4 (NM (LD)). 121320/116622 (V F2) and 206043/198185 (V F1) were found in Group 5 (NM (MD)). 202479/196028 (VI F2) and 214933/206732 (VI F1) were present in Group 6 (NM (HD)). Raw/clean reads for Group 7 (NM (MD) +Probiotic) were 203847/195350 (PF1) and 208743/200831 (PF2). Further analysis of the taxonomic annotation, community richness, and diversity was performed on the clean reads.

OTU Cluster and Taxonomic Annotation

Uparse software was used to perform the sequencing analysis, producing operational taxonomic units (OTUs) with useful tags. Relative abundance and taxonomic distribution of bacterial populations at the phylum, class, and genus levels, including unclassified sequences, were used to categorise species at each taxonomic level using the SILVA Database's SSUrRNA database.

Different microbial community structures were found among groups based on taxonomic abundance and evolutionary tree analysis. With *Prevotella* as an abundant phylum, *Lactobacillus johnsonii* became the dominating species in normal animals (Group 1: I F1 & I F2). Animals with diabetes (Group 2: II F1 & II F2) showed decreased *Lactobacillus johnsonii* and shifted towards *Spiromonas faeni* dominance. With fewer varied microbial profiles, *Spingomonas faeni* dominated the Standard drug-treated Group 3. *Prevotella predominated* in NM (LD) treated Group 4, but *Lactobacillus acidophilus and Sphingomonas predominated* in NM (MD) treated Group 5. With *Bacillus* and *Lactobacillus acidophilus* as the leading species, NM (HD) treated Group 6 showed the most diversity. While *Bacillus* and *Prevotella* dominated NM (MD + Probiotic) treated Group 7. Taxonomic relative abundance at the species level is displayed in Figure 2. In order to determine whether similar processing samples are clustered and to analyse their similarities and differences, a heatmap was also created using the abundance data of the top 35 genera of all samples (Figure 3 shows the species-level taxonomic abundance cluster heatmap). A Venn and Flower diagram was produced based on the study's findings after normalising the feature sequence table and analysing each sample's common and unique data (Figure 4).

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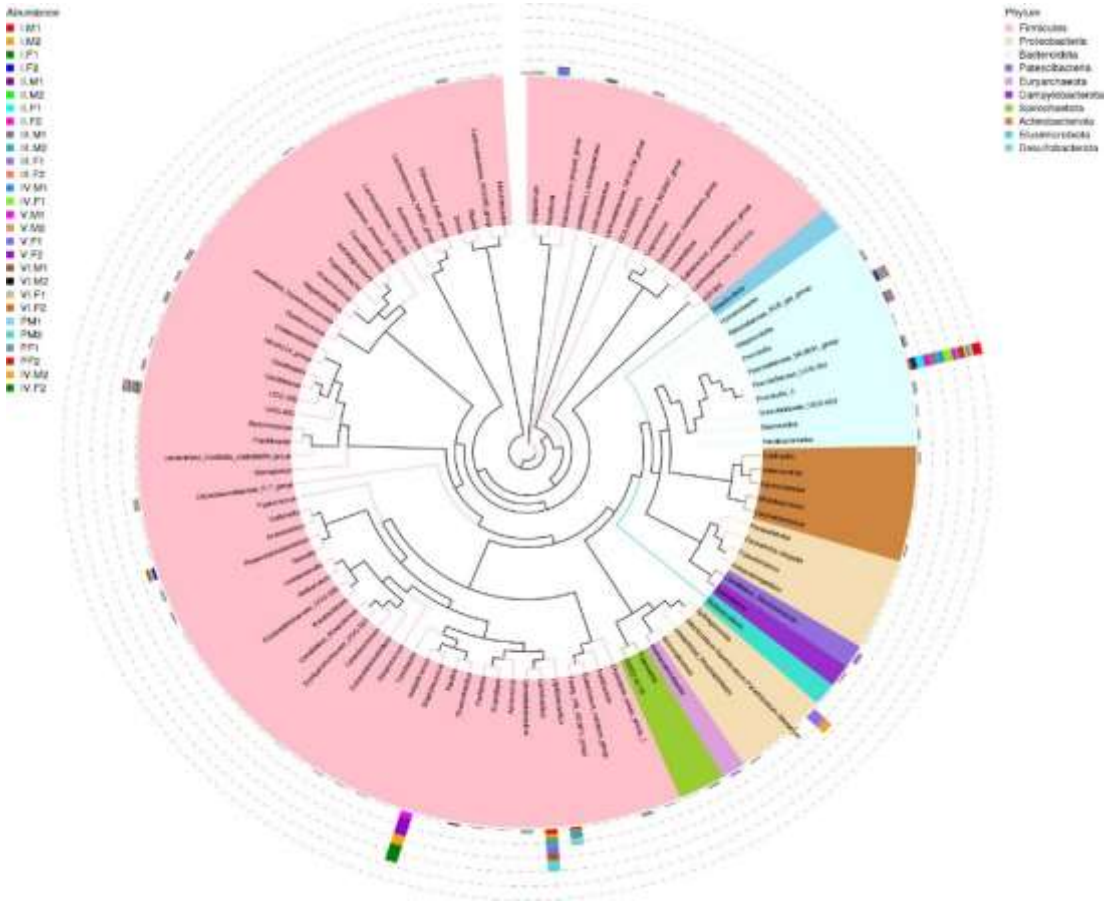

Fig. 2 Representation of evolutionary tree at the genus level of 16S data

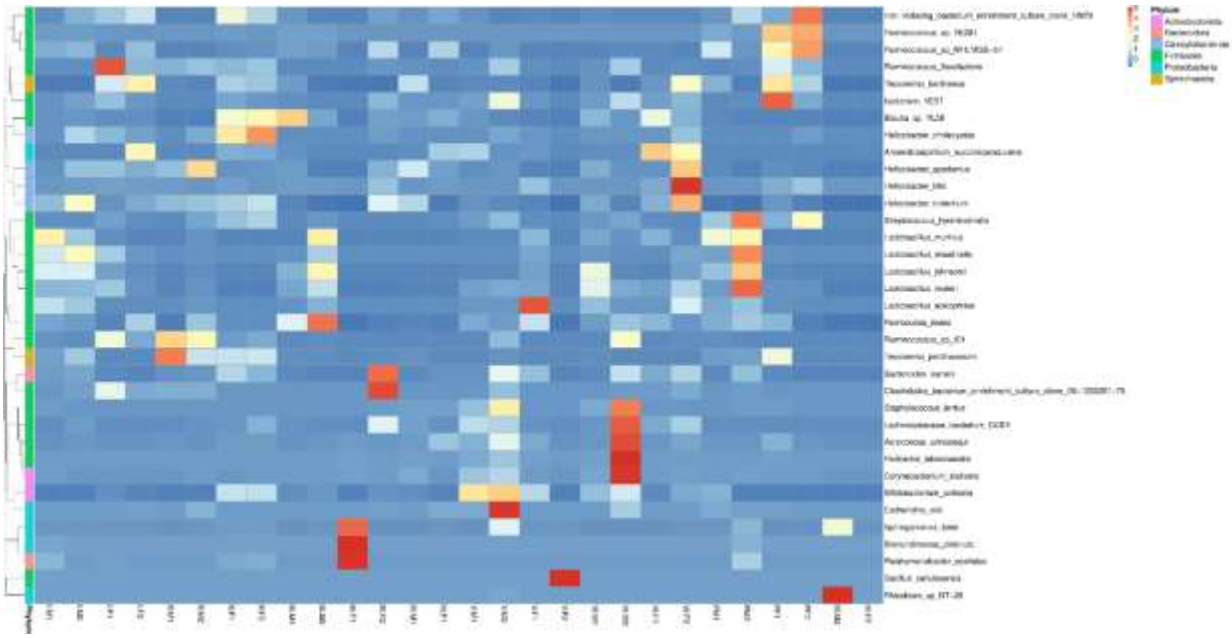

Fig.3 Species level taxonomic abundance cluster heatmap

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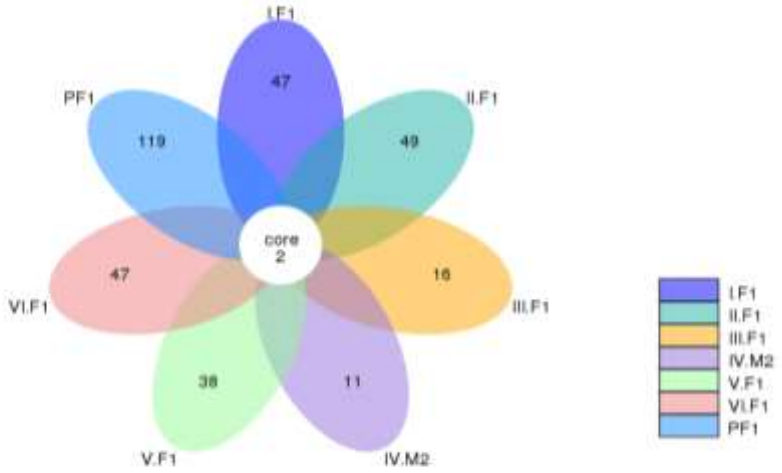

Fig. 4 Venn and Flower diagram representing common and unique data in all the

Samples

A considerable degree of bacterial variation was shown by the community richness and diversity indices derived from output normalised data (Table 4). According to the Shannon-Wiener diversity value, Group 4 had a high abundance of the species (Gaines et al., 1999; O'Keeffe, 2004). Although Group 4 animals had greater and more abundant species variety, the Simpson diversity index was close to one (1- more diversity, 0- less diversity; Shah & Pandit, 2013). Additionally, the animals of Group 6 exhibited higher diversity than the other samples, according to the ACE index and the Chao 1 index, which quantifies species richness and is sensitive to unusual OTUs (singletons and doubletons).

Table 4. The community richness and diversity indices of the gut bacteria among the rat models

Sample Name	Shannon	Simpson	Chao1	ACE
I F1	6.87	0.981	694.333	703.32
I F2	6.031	0.953	805.647	818.047
II F1	6.001	0.941	745.826	736.376
II F2	6.078	0.942	766.333	764.283
III F1	5.322	0.917	624.78	626.209
III F2	4.344	0.884	532.66	530.235
IV F1	5.129	0.894	780	789.513
IV F2	1.444	0.576	81.08	81.08
V F1	5.041	0.866	846.8	871.895
V F2	6.433	0.944	866.7	882.698
VI F1	5.052	0.918	813	805.498

Sample Name	Shannon	Simpson	Chao1	ACE
VI F2	7.049	0.981	909	921.473
PF1	5.81	0.955	584	597.2
PF2	4.454	0.867	624.9	614.492
I M1	5.965	0.951	715.68	723.749
I M2	6.521	0.963	834.038	818.887
II M1	5.902	0.949	622.509	626.208
II M2	6.05	0.954	605.533	616.9

Furthermore, a beta diversity heatmap was produced using the Weighted Unifrac and Unweighted Unifrac distances to determine the dissimilarity coefficient between pair samples. The results showed that the microbial communities of Group 4 animals are highly diverse (Figure 5). Additionally, the relative abundance of each phylum was displayed, and cluster analysis demonstrated the integration of microbial communities (Figure 6).

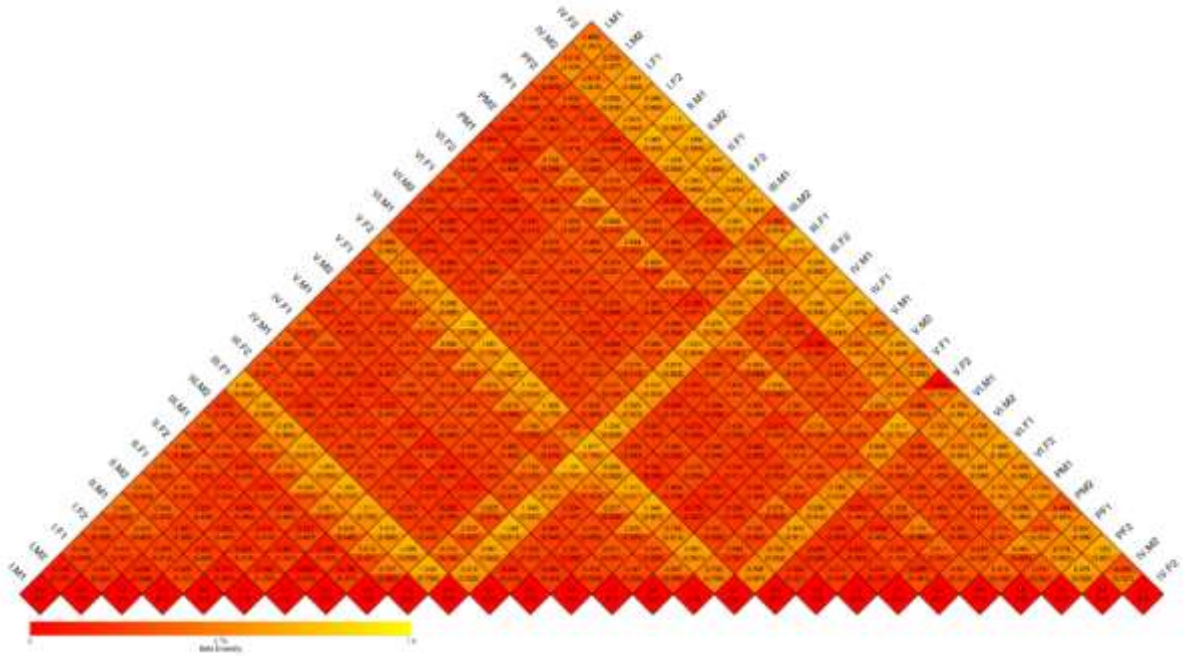

Fig. 5 Heatmap representation of Beta diversity

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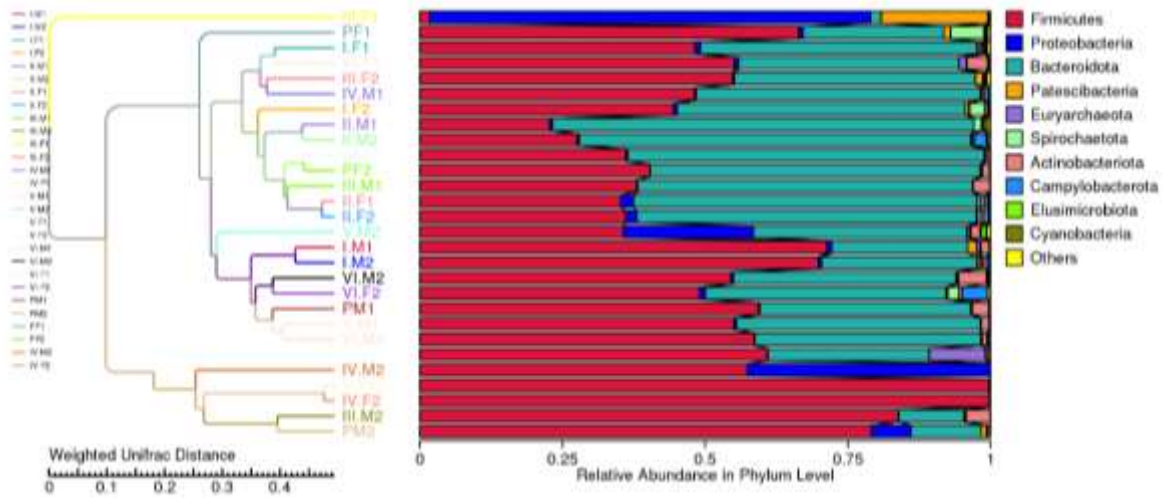

Fig. 6 UPGMA cluster tree based on Weighted Unifrac distance

PcoA Analysis

Additionally, a PCoA diagram in the first and second primary coordinates was created by clustering communities using PCoA and creating a discernible difference (Figure 7). Also, a PCA map was created in order to adequately depict the variations among samples. This map showed that Group 2 (diabetes-induced) was clearly distinct from the other groups, with Group 6 (VI F1 & VI F2) more nearly matching the anticipated pattern of healthy microbial profiles. These results were further supported by NMDS analysis, which revealed that Group 2 was clearly clustered and that Group 6 was closer to the expected distribution of healthy microbiomes. All of these findings point to Group 2's microbial communities being substantially distinct from the others, with Group 6's microbial profile more closely resembling that of a healthy condition (Figure 8). The findings of PCoA and PCA analysis were in agreement with the NMDS analysis based on the gathered OTUs (Figure 9).

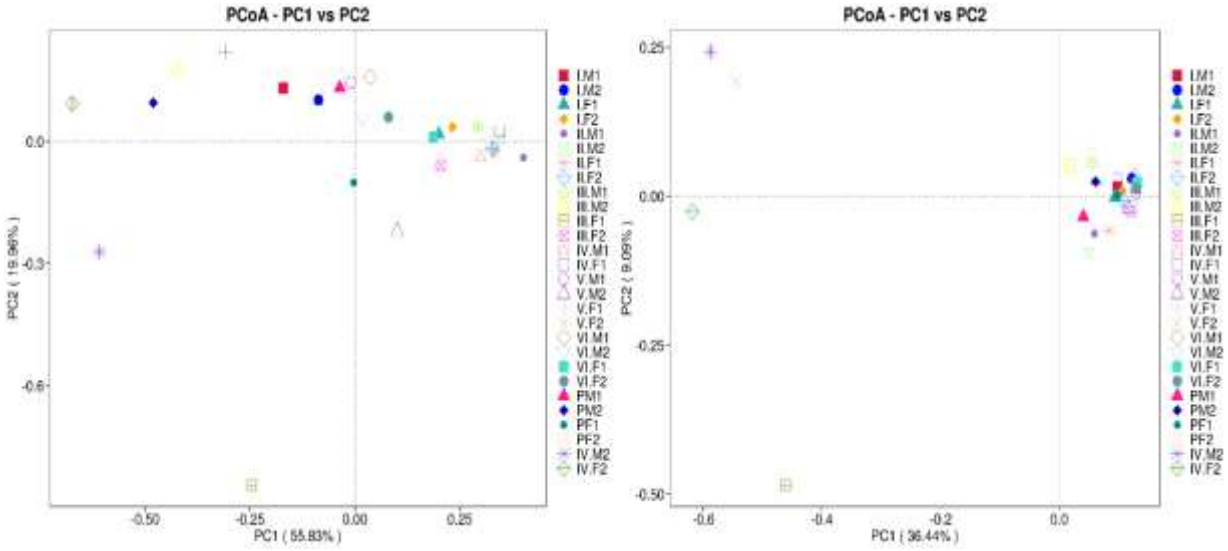

Fig. 7 PCoA based on Weighted Unifrac distance

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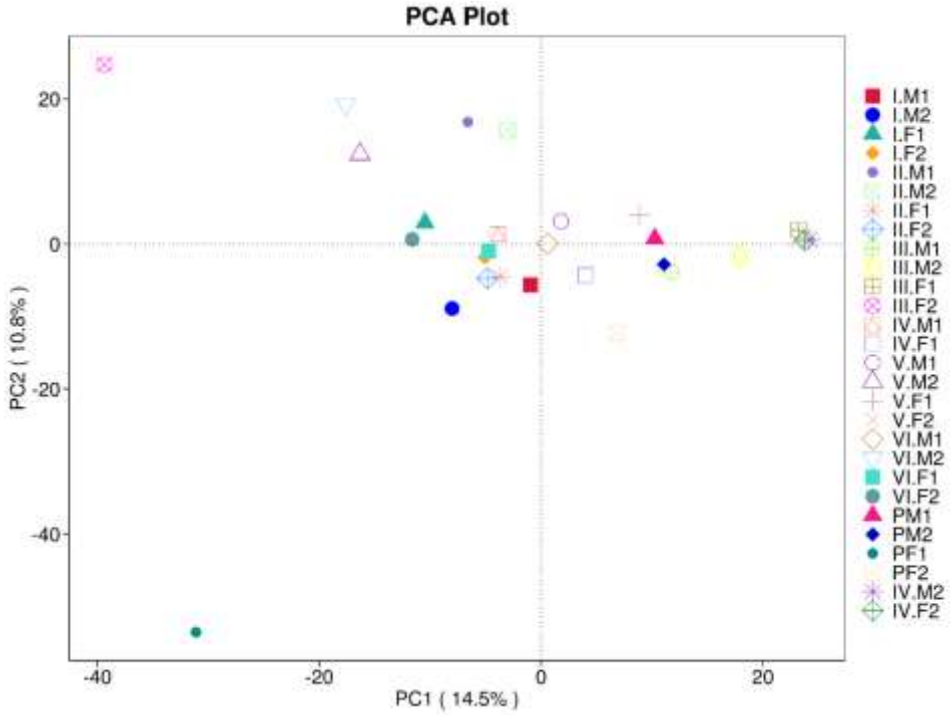

Fig. 8 PCA map of microbial community composition among the four samples

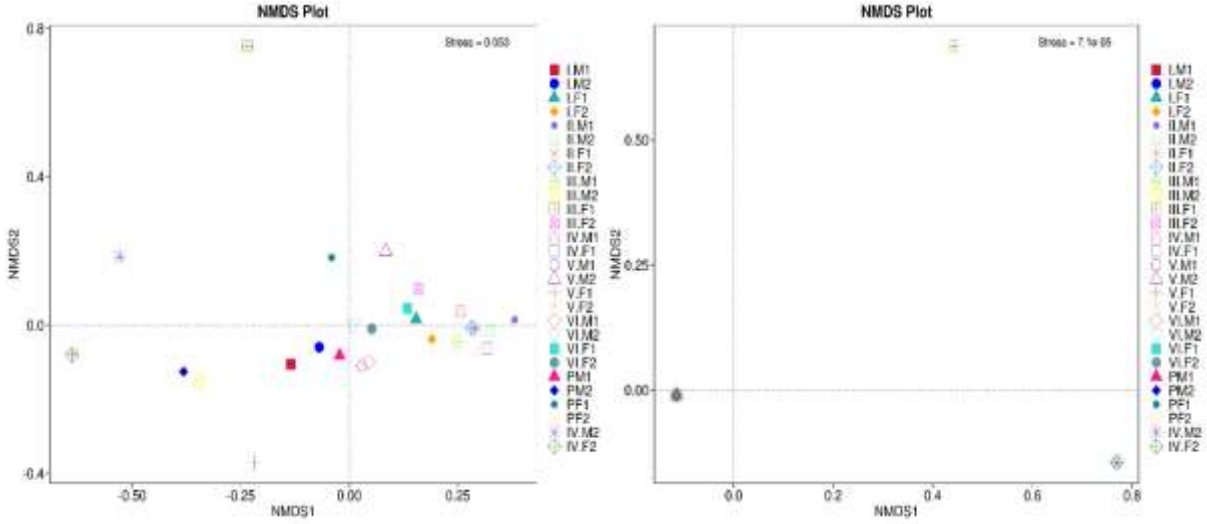

Fig. 9 Representation of NMDS analysis based on OTUs

4. Discussion

Diabetes, which is defined by high blood glucose levels, is a serious health risk that can frequently result in severe complications. Despite their effectiveness in many situations, current antidiabetic drugs might cause patients to develop drug resistance and have adverse side effects (Pokrywka et al., 2013; Kaval et al., 2013; Bhangale & Acharya, 2016). Additionally, a significant concern, especially in Type 2 diabetes, is the growth of drug-resistant diabetes, which is often accompanied by insulin resistance (Li et al., 2013). Therefore, it is essential to investigate alternative therapeutic techniques with minimum side effects and practical antidiabetic qualities.

The gut microbiome, comprising bacteria, fungi, viruses, and archaea, represents a complex and

dynamic microbial ecosystem within the human gut. Any change in the proportion of beneficial and harmful bacteria is known as Dysbiosis. This occurs in various conditions, including diabetes, obesity, liver diseases, and even in cancer.

In line with other research (Qin et al., 2012; Karlsson et al., 2013), it is established that diabetic rats' gut microbiota differed significantly from that of healthy controls. Reduced microbial diversity was seen in diabetic animals, as evidenced by an increase in potentially hazardous bacteria like *Helicobacter* and *Treponema and* a decrease in the prevalence of helpful bacteria like *Lactobacillus*. Diabetes is characterised by this dysbiosis, which has been connected to several metabolic issues (Zhao et al., 2018). Hence, it is necessary to explore drugs that can not only efficiently treat diabetes but also prevent gut dysbiosis.

Medicinal herbs have long been used to treat diabetes in traditional medical systems in India. This approach is prevalent in many Asian and African nations, where plant-based medicines are used as powerful antidiabetic drugs in traditional folk medicine (Unger & Parkin, 2010; Osadebe et al., 2013).

'Siddha', one of India's ancient Traditional Medicinal systems, has its roots in South India, especially Tamil Nadu. Siddha is a folklore Indian system of medicine that has been in practice for thousands of years for various ailments. Siddha system of medicine consists of many formulations for various ailments, including polyherbal and herbomineral formulations. 'Madhumegam' is the comparable condition for diabetes in Siddha. Many Siddha formulations, such as Madhumega Chooranam, Aavarai Kudineer, Seenthil Chooranam, Abraga Chenduram, Naaval Kottai Chooranam, have been the most in practice for diabetes management. This study aimed to evaluate the effects of one Siddha herbomineral formulation, Neeradimuthuvallathy Mezhugu, on the intestinal microbiota of diabetic rats. The results showed that NM can modify the gut microbial environment and offer new insights into the changes in the gut microbiota associated with diabetes.

The intestinal microbiota composition of diabetic animals was markedly altered by treatment with the test drug NM. Interestingly, higher Shannon and Simpson indices and greater richness (Chao1 and ACE) indicated that particular treatment groups had more microbial diversity. Additionally, *Lactobacillus* and *Bacillus*, two beneficial bacteria that are known to be essential for preserving gut health, controlling glucose metabolism, and having anti-inflammatory properties (Chen & Zhang, 2023; Fusco et al., 2023), were enriched as a result of the NM treatment. On the other hand, these groups had much lower levels of potentially pathogenic microorganisms. These results imply that by encouraging the growth of advantageous bacteria and inhibiting the growth of harmful ones, the NM may positively affect the gut microbiota.

Furthermore, Group-6 (NM(HD)) had a more noticeable influence on the gut microbiota than Glibenclamide, a common anti-diabetic drug that successfully regulates blood glucose levels. Limited microbial diversity and a predominance of potentially less advantageous taxa were observed in Group 3 (Standard drug). Hence, it is clear that the Siddha formulation in Group 6 (NM (HD)) affects the intestinal microbiome.

Prebiotic effects are one possible route through which the Siddha formulation can specifically promote the growth of good bacteria (Carding et al., 2015). Furthermore, the Siddha medication may have anti-inflammatory properties in the stomach, which would improve the conditions for the growth of good bacteria due to abundant phytochemicals in NM and thereby possess potential prebiotic and probiotic properties.

The results of this study have important therapeutic ramifications. Siddha medicine's beneficial effects on the gut flora point to its potential as a cutting-edge diabetic treatment strategy. This work offers a solid basis for further investigations into the creation of microbiome-based treatments, such as probiotics and prebiotics, as supplemental treatments for diabetes. These results also highlight the

significance of considering personalised medicine strategies, which could modify the choice of treatment therapies according to each patient's unique gut microbiome composition.

More research involving human participants is essential to confirm these results. Furthermore, even though this study sheds light on the taxonomic makeup of the gut microbiota, more functional analyses, such as metagenomics and metatranscriptomics, are required to comprehend the gut microbiome's functional potential and how its metabolic processes react to treatment with Siddha medicine.

To sum up, this study offers strong proof that a particular Siddha herbal composition can dramatically alter the intestinal microbiome of diabetic rats. The observed rise in microbial diversity, enrichment of helpful bacteria, and decrease in potentially dangerous bacteria suggest a favourable effect on gut health and potential improvement in metabolic outcomes in diabetes. These results highlight the need for more investigation into the therapeutic potential of Siddha medicine and the function of gut microbiota in the treatment of diabetes.

5. Conclusion

Using 16S rRNA gene sequencing, this study examined the effects of Neeradimuthuvallathy Mezhugu (NM), a formulation of traditional Siddha medicine, on the intestinal microbiota of diabetic rats. A common anti-diabetic medication called Glibenclamide was used to compare the effects of NM. To sum up, our work offers strong proof that the ancient Siddha herbal combination Neeradimuthuvallathy Mezhugu (NM) can considerably alter the intestinal microbiota of diabetic rats. NM had a more noticeable good effect than glibenclamide, a common anti-diabetic medication, by increasing the number of beneficial bacteria like *Lactobacillus* and *Bacillus* and decreasing the number of harmful ones. This implies that by enhancing gut health and maybe reducing the metabolic problems linked to the condition, Siddha treatment may present a viable strategy for managing diabetes. To completely comprehend the therapeutic potential of NM and its influence on the gut microbiota in the management of diabetes, more investigation is necessary, including human clinical trials and comprehensive mechanistic studies.

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