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AMELIORATION OF GUT STABILITY OF DIABETIC PATIENTS USING *NAUCLEA LATIFOLIA* FRUIT EXTRACTS

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ABSTRACT

Diabetic patients are typically associated with gut dysbiosis. However, little or no research has been conducted on the restoration of gut microbiota in diabetic patients in Nigeria, particularly Anambra State. This study focused on the amelioration of gut stability in diabetic patients using *Nauclea latifolia* fruit extracts (NLFE). Forty stool samples were collected (20 from diabetic patients and 20 from non-diabetic subjects). Comprehensive digestive stool analysis (CDSA) was performed using standard plate counts and instrumental techniques. Unique bacterial isolates from diabetic patients were characterized. An in vivo human trial assessed the effect of NLFE on gut stability. Data were analyzed using one-way ANOVA and Student's t-test at 95% confidence level. CDSA revealed decreased counts of *Clostridium* (CC), *Bacteroides* (BAC), and *Lactobacillus* (LC), with a significant decrease ($p < 0.05$) in *Bifidobacterium* counts (BC) among diabetic patients. Following NLFE administration, CC, BAC, BC, and LC showed progressive restoration, reaching significance ($p < 0.05$) after six months. NLFE demonstrated pronounced activity, restoring 80% of gut stability in diabetic patients. This study provides the first evidence in Anambra State, Nigeria, that *Nauclea latifolia* fruit extract effectively restores gut microbiota in diabetic patients, offering a potential dietary intervention for diabetes-associated dysbiosis.

Keywords: Microbiota, Dysbiosis, Diabetes, *Nauclea latifolia*

1.0 INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by persistent hyperglycemia, which may result in long-term complications leading to damage to many of the body's systems, especially kidneys, nerves, eyes, and blood vessels and also has become a major public health concern. Autoimmune type 1 diabetes (T1D) and insulin resistant type 2 diabetes (T2D) are the two main types. A combination of genetic and environmental factors contributes to the development of these diseases.

Type 1 diabetes (T1D) is a chronic autoimmune disease characterised by the immune-mediated destruction of insulin-producing pancreatic beta cells, usually occurring in children and young adults, (Gulden *et al.,* 2015, Atkinson *et al.,* 2014, Abdellatif and Sarvetnick, 2019). Although there is still some uncertainty about the aetiology of T1D, it is currently considered a multifactorial autoimmune disorder involving both genetic predisposition and environmental factors (Pociot and McDermott, 2002, Esposito *et al.,* 2019). With the introduction of high-throughput sequencing, the structure of microflora can be analysed more comprehensively than before (Forde and O'Toole, 2013). Intestinal microbiota, known as the "human second genome" (*Qin et al.,* 2010), can coevolve with their host in a symbiotic relationship by combating pathogenic organisms (Stecher and Hardt, 2011), assisting in food digestion (Shreiner *et al.,* 2015), maintaining the integrity of the intestinal epithelia (Natividad *et al.,* 2012), and promoting immunological development (Natividad *et al.,* 2012, Dave *et al.,* 2012).

In the past decade, there has been growing evidence suggesting that gut dysbiosis may be a major contributor to T1D development (Vaarala, 2013). A variety of studies have identified differences in the gut microbiota of healthy subjects and T1D patients (Bibbò *et al.,* 2017). In addition, growing evidence from well-controlled intervention studies in rodent models has supported the causative association between gut dysbiosis and T1D pathogenesis. The use of probiotics, the use of antibiotics, fecal microbiota transplantation, and diet intervention are the methods commonly used in these studies to alter the composition of gut microbiota. It has been proposed that the altered intestinal microbiota may impact T1D pathogenesis by increasing gut permeability (Bosi *et al.,* 2006), facilitating intestinal inflammation (Westerholm-Ormio *et al.,* 2003), and

disturbing immunological maturation (Bendtsen *et al.,* 2015, Brown *et al.,* 2019). Nevertheless, given the paucity of well-controlled studies in humans owing to the lack of corrective methods for confounding factors, gut microbiota as a causal factor leading to the progression of T1D remains speculative. Once the causative relationship between gut microbiota and T1D development is confirmed and the related pathophysiological mechanisms are delineated, the gut microbiota will be a novel area to explore for new preventative or therapeutic strategies for T1D. Discovery of a clear association between gut dysbiosis and T1D is of significant clinical importance as microbiota-based interventions such as probiotics can reduce or even prevent the burdensome requirement of injected insulin.

2.0 MATERIALS AND METHODS

Sample Collection and Transportation

Stool and blood samples were collected by sampling technique and analyzed in the Microbiology Laboratory unit of Nnamdi Azikiwe University, Awka. Ripe fruits of *Nauclea latifolia* were collected from Ilorin, Kwara State, Nigeria. They were identified and authenticated by Prof. I.B. Enweani. The fruits were chopped into small pieces and air-dried at room temperature. They were then pulverised using a blender (Supper master blender, model: SMB-2977) to yield fine powdered material. Then, 45 g of the powdered sample was mixed with 8.5 litres of clean water, boiled for 20 minutes and allowed to cool. The resulting extract was filtered and kept refrigerated in air-tight containers. For six months, the filtered extract was given twice a week to both the diabetic patients and the healthy individuals (Enweani, 2020). Their blood glucose levels were examined morning (fasting) and night (random) for each day.

2.1 Comprehensive Analysis of Gut Bacteria using the Stool Samples

2.1.1 Complete digestive gut analysis: This was carried out using standard plate counts of *Clostridium*, *Bifidobacterium*, *Lactobacillus* and *Bacteriodes* as described in the study published by Marco *et al.* (2017).

2.2 Characterization of the major Bacterial Isolates Associated with the Gut

2.2.1 Purification of the isolates : The plates that showed discrete colonies were selected after 24 h, and aseptically streaked each colony on sterile plates (90mm×15mm) containing nutrient agar (BIOTECH) prepared according to the manufacturer's description. The streaked plates were placed in a bacteriological

incubator in inverted positions and incubated at 35 ± 2^0C for 24 h as described in Cheesbrough (2010).

2.2.2 Characterization of the pure isolates: The pure isolates were characterized using the morphological, biochemical and molecular characteristics as described by Frank and Robert (2015) and (Cheesbrough, 2010).

2.3 Effect of *Nauclea latifolia* fruit extracts on gut stability

Preparation of *Nauclea latifolia* fruit extracts: The fresh fruits of *Nauclea latifolia* was collected and it was appropriately authenticated. The fruit samples were ground to paste form using sterile electric grinder (LE Max/ LXB 242). Twenty grams of the ground sample was macerated with distilled water for 72 h. Whatman No 1 filter paper was used to filter the mixture. The extract was concentrated by evaporating to dryness at room temperature in a steady air current (Iheukwumere *et al.,* 2018). Then 40 g of the extract was dissolved in phosphate buffer saline (PBS) and made-up to 2000 mL using the PBS in order to obtain 20 mg/mL concentration

Acute toxicity: The albino Wistar rats were monitored for 72 h for mortality cases as described in the work published by Iheukwumere *et al*. (2018). A total of 16 albino Wistar rats were used for this study. The rats were grouped into two groups. Each group contained 8 rats each. The test rats were orally administered 1.0 g/ kg (tenfold of normal administration) of the prepared extracts whereas the other group (control group) was giving ordinary distilled water as normal control. The rats in each group were monitored for 72 h during which the acute toxicity was determined after 72 h.

2.4. Determination of gut stability: A total of 20 diabetic patients, 10 males and 10 females from different hospitals were recruited for this study. The patients that were already on drugs were excluded in this study. This was a single- blinded study. The prepared extract was orally administered by the diabetic patients, ten milliliters, two times per day, for period of 4 months. Then stool samples from each patient was collected every month and analyzed for complete microbial gut analysis as already described in this study above

2.5. Statistical Analysis

The data generated were analyzed using one-way Analysis of Variance (ANOVA) at 95% confidence level, and compared using student “t” test.

3.0 RESULTS

Gut Stability and Blood Glucose Level among Diabetic and Non-diabetic Subjects

The comprehensive digestive analysis (CD&A) study of the gut revealed decrease in total *Clostridum* counts (CC), total *Bifidobacterium* counts (BC), total *Lactobcillus* counts (LC) and total *Bacteroides* counts (BAC) as shown in Table 1. It was observed that CC, BC, LC and BAC were higher in females than males, and the decreased in BC was statistically significance ($P < 0.05$). The study also revealed that BAC > CC > LC > BC.

Characteristics of the Predominant Bacterial Isolates in the Gut of Diabetic Patients

The cultural and morphological characteristics of the bacterial isolates showed that the isolates had raised elevations, smooth edges, non-motile and non-pigmentation producers. The isolates did not require oxygen for their growth (anaerobes) and they were spore formers. Isolates R and S differed from T by being Gram negative rods. Isolate R differed from S by forming cream / white colonies on nutrient agar as shown in Table 2.

The biochemical characteristics of the isolates are shown in Table 3. The isolates were catalase, indole and urease negative, and partially utilized citrate as their sole source of carbon. Isolates R and S were gelatin and esculin positive whereas isolate T was negative to gelatin and esculin hydrolysis. The isolates differed mainly in their abilities to utilize different fermentation sugars and sugar alcohols as shown in Table 3.

Effect of *Nauclea latifolia* Fruit Extract on Gut stability of Diabeic Patients

The study revealed that the decreased in the total *Clostridium*, *Bifidobacterium*, *Lactobacillus* and *Bacteroides* seen in the diabetic patient showed progressive increased in every two months among the diabetic patients that were taking *Nauclea latifolia* fruit extracts (NLFE) as shown in Table 4.

The study showed progressive restoration of the gut microbiota of the diabetic patients taking NLFE, and the study became statistically significance ($P < 0.05$) after six months for *Bifidobacterium* counts.

Table 1: Comprehensive digestive analysis (CD&A) study

Mean Counts (Log CFU/g)

Subject	*Clostridium* Counts	*Bifidobacterium* Counts	*Lactobacillus* Counts	*Bacteroides* Counts
NM	4.48 ± 0.21	4.08 ± 0.11	4.43 ± 0.07	4.58 ± 0.07
DM	3.82 ± 0.17	1.88 ± 0.03	3.22 ± 0.11	4.01 ± 0.11
NF	4.52 ± 0.12	4.12 ± 0.21	4.45 ± 0.14	4.62 ± 0.17
DF	3.88 ± 0.07	2.05 ± 0.03	3.51 ± 0.11	4.11 ± 0.07

NM – Normal Male; DM – Diabetic Male, NF – Normal Female; DF – Diabetic Female

Table 2: Cultural and morphological characteristics of the most abundant isolates

Parameter	R	S	T
Colour nutrient Agar in Anaerobic condition	Cream / white	Colourless	Gray white / colourless
Edge	Smooth	Smooth	Smooth
Elevation	Raised	Raised	Raised
Pigmentation	-	-	-
Spore	+	+	+
Gram Reaction	-	-	+

Cell morphology	Rods / ovoid	Rods	Rods
Motility	-	-	-
Oxygen Requirement	-	-	-

Table 3: Biochemical characteristics of the bacterial isolates

Parameter	R	S	T
Catalase	-	-	-
Indole	-	-	-
Citrate	+ /-	+ / -	+ / -
Urease	-	-	-
Gelatin	+	+	-
Esculin	+	+	-
Glucose	+	+	+
Fructose	+	+	-
Lactose	+	+ / -	+
Xylose	+ / -	+ / -	-
D-mannose	+ / -	-	+ / -
Maltose	+	+	+ / -
Mannitol	+ / -	+ / -	-
Sorbitol	+ / -	+	-
Sucrose	+	+ / -	+

Table 4: Comprehensive digestive analysis (CD&A) study

Mean count (Log CFU/g)	2 Months			4 Months			6 Months		
	N	D	E	N	D	E	N	D	E
Clostridium counts	4.49 ± 0.21	3.87 ± 0.17	4.12 ± 0.17	4.48 ± 0.27	3.84 ± 0.11	4.22 ± 0.41	4.51 ± 0.19	3.64 ± 0.12	4.29 ± 0.11
Bifidobacterium ccounts	4.09 ± 0.37	1.86 ± 0.11	2.96 ± 0.22	4.06 ± 0.17	1.84 ± 0.11	3.08 ± 0.14	4.08 ± 0.12	1.83 ± 0.17	3.24 ± 0.11
Lactobacillus counts	4.41 ± 0.19	3.11 ± 0.11	3.72 ± 0.33	4.43 ± 0.14	3.07 ± 0.51	3.91 ± 0.14	4.42 ± 0.21	3.02 ± 0.11	3.97 ± 0.33
Bacteroides counts	4.56 ± 0.81	4.02 ± 0.33	4.19 ± 0.67	4.58 ± 0.17	4.04 ± 0.67	4.28 ± 0.11	4.56 ± 0.33	4.01 ± 0.14	4.39 ± 0.19

Discussion

The decreased in total *Clostridium* counts (CC), total *Bifidobacterium* counts (BC), total *Lactobacillus* counts (LC) and total *Bacteroides* counts (BAC) associated with the guts of diabetic patients corroborated with the findings of many researchers (Caselin and Sabatino, 2019; Jelinek and Tweedie, 2018; Rayner and Horowitz, 2013; Verhagen and Nieuwdorp, 2018). The study generally highlighted a total decreased in the gut microbiota and loss of gut vital microbiota such as *Bifidobacterium* and *Lactobacillus* as reported by Rayner and Horowitz (2013).

The occurrence of unique anaerobic bacteria; *Segatella copris* strain YE2 (SCY2), *Prevotella jejuni* strain F0697 (PJF7) and *Romboutsia* species strain 13432 (RS2) among the diabetic patients signifies deviation from the normal microbiota encountered in non-diabetic individuals. The occurrences of these unique bacterial isolates could be attributed to gut ecosystem destabilization, loss of vital gut microbiota associated with the gut of diabetic patients. Several researchers (Jelinek and Twedie, 2018; Verhagen and Nieuwdorp, 2018; Casella Sabatino, 2019; Garcia and Nieto, 2020; Rao and Alhaji, 2020) have worked on various diabetic gut and were able to detect unique bacterial isolates that altered the gut microbiota, some of their findings agree with the present report in this study.

The progressive restoration of the gut microbiota and ecosystem in every two months interval associated with the diabetic patients taking NLFE observed among the experimented subjects supported the findings of many researchers (Nieuwdorp and Verhagen, 2018; Casella and Sabatino, 2019; Garcia and Nieto, 2020; Rao and Alhaji, 2020) who were able to observe progressive restoration of gut of diabetic patients after administering different restoration compounds. Further studies reviewed that majorities of the restoration achieved by the researchers were carried out using probiotics and prebiotics, but in the present study, NLFE was used. There were increased in the total counts of notable bacterial isolates (CC, BC, LC and BAC) among the diabetic patients that were taking NLFE, and these became significance after six months of taking NLFE.

CONCLUSION

The comprehensive digestive analysis study of the gut of diabetic patients revealed decrease in total *Clotridium* counts (CC), total *Bifidobacterium* counts (BC), total *Lactobacillus* counts (LC) and total *Bacteroides* counts (BAC). There was

progressive restoration of gut microbiota among the diabetic patients that were taking NLFE, and these became significance after six months.

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