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Association of *Proteobacteria* and *Bacteriodetes* with Obese Related Type-2 Diabetes Mellitus

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Metabolic conditions such as Type2 diabetes mellitus (T2DM) and obesity have become worldwide public health important. Numerous evidences indicate that gut *Proteobacteria* and *Bacteriodetes* are associated with these co-morbidities. Thus, the gut microbiota serves as a promising target for prognosis of metabolic disorders. The aim of this study is to evaluate the role of gut *Proteobacteria* and *Bacteriodetes* on obese related Type 2 diabetes mellitus. The gut microbiota signature of 10 adults was studied using 16S rRNA sequencing targeting V_3–V_4 hypervariable regions and obtained data was analyzed using Statistical Package for the Social Science (SPSS version 26). Result: The Pearson correlation analysis showed that phyla

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Bacteriodetes was significant positive when correlated with Body mass index (BMI) (r = 0.666, p = 0.002*), followed by phyla *proteobacteria* (r = 0.464, p = 0.045*), *Firmicutes* versus BMI (p>0.05), *Actinobacteria* versus BMI (p>0.05), while *Firmicutes* versus Glycated hemoglobin(HbA1c) (p>0.05), *Bacteriodetes* versus HbA1c (p>0.05), *Proteobacteria* versus HbA1c (p>0.05), *Actinobacteria* versus HbA1c (p> 0.05). Conclusion: The study revealed the abundance of phyla *Proteobacteria* and phyla *Bacteriodetes* were significantly associated with obese related type 2 diabetes mellitus. Although, these Phyla/ taxa showed no significant correlation with Hb1Ac in obese related type 2 diabetes mellitus.

Keywords: Type2 diabetes; obese; Proteobacteria; Bacteriodetes; glycated hemoglobin; body mass index.

1. INTRODUCTION

"Proteobacteria is the most abundant phyla and phenotypically most diverse divisions within the domain bacteria" [1]. "Phylogenetic trees of 84 *Proteobacteria* were observed using single gene-based phylogeny with 16S rRNA genes and protein sequences of 85 conserved genes, whole genome-based phylogenetic tree using CVtree3.0, amino acid Identity matrix tree, and other conserved genes" [2].

The previous studies have shown that the change of microbiota associated with metabolic diseases like type-2 diabetes [3,4]. Their findings indicate a significant high abundance of *Enterobacteriaceae* family together with phylum *Proteobacteria*), in the T2DM group when compared to the control groups [3]. *Proteobacteria* is found in low abundant in healthy gut microbiota, though, it happens to increase in abundant in the cases of metabolic disorders [5,6]. The class of these *proteobacteria* possess lipopolysaccharide (LPS, endotoxin) molecules that prone to cause the inflammatory responses [7]. LPS induces the secretion of interleukin 8 (IL-8) which is the major chemokine involves in the inflammatory responses, which, in turn, change the tight junctions and cause the impairment of epithelial integrity inside the enterocytes [8].

Currently, members of the phylum *Bacteriodetes* are divided into four main classes that include: *Bacteriodia*, *Flavobacteriia*, *Sphingobacteriia*, *Cytophagia* [9]. "As members of polysaccharide-degrading consortia, they attribute to the release of energy from dietary fiber and starch, and they are major source of propionate" [10]. "While, other members of this group have some activities that may help to suppress inflammation, but they also have the potential to promote inflammation and some are known to be opportunistic pathogens" [11,12]. However, this study was carried out to understand the association of gut *Proteobacteria* and *Bacteriodetes* with obese T2DM patients in Nigerian population.

2. METHODOLOGY

2.1 Study Design

This is a cross-sectional study to determine the metagenomic study of obese related Type2-diabetes mellitus in Nigeria. One hundred and twenty (120) subjects were enrolled by convenience sampling for this study. They comprise one hundred and ten (110) T2DM subjects who were Diabetic clinic attendees at Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria and Ten (10) non-DM health workers. Both test and control groups were aged between 20-80 years and included male and female subjects. This study covered a period of twelve months.

2.2 Inclusion Criteria

Subjects for the study include male and female subjects between the age of 20-80 years. The subjects that were confirmed to be diabetic and not on antibiotic drug for two weeks were enrolled. There was no diet restriction among the T2DM subjects. The subjects were not placed on any diets.

2.3 Exclusive Criteria

Non-diabetic, pregnant females and vegetarians were excluded and subjects within the age bracket of 10 -19 years were not recruited.

2.4 Stool sample collection

Stool specimens obtained from the subjects were transferred into separate ubiome sample

tubes following ubiome sample collection instructions. These samples were stored at ambient temperature before being shipped to ubiome in San Francisco (USA) for processing. All participants were interviewed and their ages, gender, weights, heights, ethnicity, diet, history, random glucose levels were ascertained.

2.5 Anthropometric Measurement

The anthropometric measurements used for Body Mass Index were Weight and Height. Weight and height were measured using a standard beam balance scale and a stadiometer respectively. Body Mass Index (BMI) was calculated as weight in kilogram divided by height squared in meters.

BMI (kg/m^2) = Weight (kg) divided by Height (m^2)

2.6 Blood Sample Collection and Processing

Venous blood sample, (3ml) volume was collected from each subject using 5.0ml sterile disposable syringe and dispensed into 3ml EDTA sample containers and labelled with the subject's name, age and sex.

2.7 Principle of Glycohemoglobin

A hemolyzed preparation of the whole blood is mixed continuously for 5minutes with a weak binding cation-exchange resin. During this time, HbA1C binds to the resin. After the mixing period; a filter is used to separate the supernatant containing the glycohemoglobin from the resin. The percent glycohemoglobin is determined by measuring absorbance at 415nm of the glycohemoglobin fraction and the total hemoglobin fraction. The ratio of the two absorbance gives the percentage glycohemoglobin.

2.8 Procedure Outline

2.8.1 Hemolysate preparation

A 500 µl volume of lysing reagent was dispensed into 3 tubes labeled; standard, control and sample. A 100 µl volume of the well-mixed serum sample, standard, and control were placed into the appropriately labeled tubes and properly mixed. They were allowed to stand for 5minutes.

2.8.2 Glycohemoglobin preparation

Three (3.0) ml of glycohemoglobin cation-exchange Resin was dispensed into glass tubes labeled standard, Control and sample. A 100µl volume of the hemolysate was added (from hemolysate preparation step) into the tubes. The filter separator was positioned in the tubes so that the rubber sleeve was appropriately 1 cm above the liquid level. The tubes were placed on the rocker and mixed continuously for 5minutes. The tubes were removed from the rocker. The filter separator was pushed into the tubes until the resin was firmly packed. The supernatant was poured into another tube and then poured directly into a curvette for absorbance measurement. The instrument was adjusted to zero absorbance at 415nm with deionized water as the blank (Wavelength range: 390-420nm). The absorbance values for standard, control and sample were read and these readings were values for glycohemoglobin.

2.8.3 DNA extraction, PCR amplification and sequencing

"DNA was extracted individually from all subjects' stool samples using QiaAMP mini stool kit (Qiagen, Valencia, CA, USA)" [13]. "To assess the composition and diversity of the subjects' gut bacterial communities, we were able to use only 19 T2DM samples out of 110 T2DM samples and 10 non-DM samples with intact and good quantity of DNA to conduct high-throughput sequencing of the V4 region of the 16S rRNA gene" [14]. "PCR amplification was performed on this region in triplicate using the 515f/806r primer pair with unique 12 bp barcodes specific to individual samples and combined the resulting product for each sample. PCR product was quantified using the Pico Green dsDNA assay, and the samples' bar-coded amplicons were combined in equimolar concentrations. Sequencing was performed on an Illumina MiSeq instrument to produce 150 bp sequences at the Ubiome center in San Francisco, USA".

2.9 Statistical Analysis

This study was analyzed using statistical package of social science (SPSS) version 26 and Pearson correlation was used to calculate the significant differences of Body Mass Index (BMI) and glycohemoglobin of obese related Type2 diabetes mellitus subjects. Stacked bar

was also used to compare the relative abundance between obese-T2DM subjects.

3. RESULTS

This study revealed the relative abundance of phyla *roteobacteria, Bacteroidetes* and *Firmicutes* were positively correlated with Body mass index as follows *Proteobacteria* versus BMI (r = 0.363, p = 0.045*); *Bacteroidetes* versus BMI(r = 0.666, p = 0.002*); *Firmicutes* versus BMI (r = 0.363, p = 0.127) whereas that of *Actinobacteria* demonstrated a negative correlation(r = -0.292, p = 0.225). It was also found that phylum *Bacteroidetes* was significantly positive with BMI (Table 1).

It was observed that the relative abundance of *Proteobacteria, Actinobacteria* and *Firmicutes* was positively correlated with Glycated hemoglobin as follows *Proteobacteria* versus HbA1C (r = 0.031, p = 0.901); *Actinobacteria* versus Hb1AC (r = 0.171, p = 0.483); *Firmicutes* versus Hb1AC (r = 0.176,p = 0.471) whereas that of *Bacteroidetes* showed a negative correlation (r = -0.042, p = 0.864) (Table 2).

Five phyla are common to all the obese related type-2 diabetes subjects, indicating higher abundance of *Firmicutes* in subject 9 followed by subject 10, subject 2 , subject 8 and subject 4 respectively, and more abundance of *Proteobacteria* was found in subject 8 > subject 9 > subject3 > Subject 2 > subject 7> Subject 4 (Fig. 1; Fig. 2). Subject 1 had more abundance of *Bacteriodetes* followed by subject 2 and subject 3 respectively (Fig. 1; Fig. 2).The higher abundance of *Actinobacteria* was found in subject 4 followed by subject 1, subject 10, subject 9, subject 3 and subject 2 respectively (Fig. 1; Fig. 2). The higher abundance of *Verrucomicrobia* was found in subject 4 followed by subject2 respectively. The abundance of *Deterribacteres* were found in subject 1, subject 2 , subject 3 , and subject 4 respectively. Although, *Deterribacteres* was not found in subject 10, subject 5, subject 6, subject 7, subject 8, and subject 9 respectively (Fig. 1; Fig. 2).

The high proportion of *Tenericutes* was found in subject 7 followed by subject 3 and subject 2 respectively (Fig. 1; Fig. 2). Subject 7 had More abundance of *Cyanobacteria* followed by subject 2, subject 4 and subject 3 respectively (Fig. 1).

4. DISCUSSION

This study revealed that relative abundance of phyla *Proteobacteria, Bacteriodetes* and *Firmicutes* were positive correlated with Body mass index, whereas that of *Actinobacteria* demonstrated a negative correlation. It was also found that *Bacteriodetes* and *Proteobacteria* significantly positive with BMI. It was observed that the relative abundance of *Proteobacteria, Actinobacteria* and *Firmicutes* were positive correlated with Glycated hemoglobin, whereas that of *Bacteroidetes* showed a negative correlation. This study agreed with the previous studies that showed high abundance of *Firmicutes* among obese related T2DM but also lower level of *Bacteriodetes* [4,7]. “The authors of the studies concluded that carbohydrate metabolism is the predisposing factor. They observed that the microbiota of obese individuals are more heavily enriched with bacteria of the phylum *Firmicutes* and less with *Bacteriodetes*. The present study showed that the relative abundance of phyla *Proteobacteria,* and *Bacteriodetes* was positively correlated with obese related Type 2 diabetes mellitus. The *Firmicutes* are well known for fat digestion, and their increased abundance is also known to be associated with obesity” [15].

Out of 10 T2DM subjects, it was found that only subject 2 and subject 4 had high abundance of *Firmicutes*, *Bacteriodetes*, *Verrucomicrobia*, *Actinobacteria* *Cyanobacteria*, and *Deferribacteres* respectively. “Similarly, *Bacteroidetes* play a crucial role in producing short-chain fatty acids (SCFAs), and *Actinobacteria* execute a key role in the biodegradation of resistant starch. It is also suggested that *Firmicutes* and *Bacteriodetes* enhance the monosaccharide uptake from the host gut, which elevates the production level of hepatic triglycerides, thereby resulting in insulin resistance”. “This change might contribute positively to low-grade inflammation mainly achieved through activation of SCFA-linked G-protein-coupled receptors (GPCR), thus leading to metabolic disorders”.

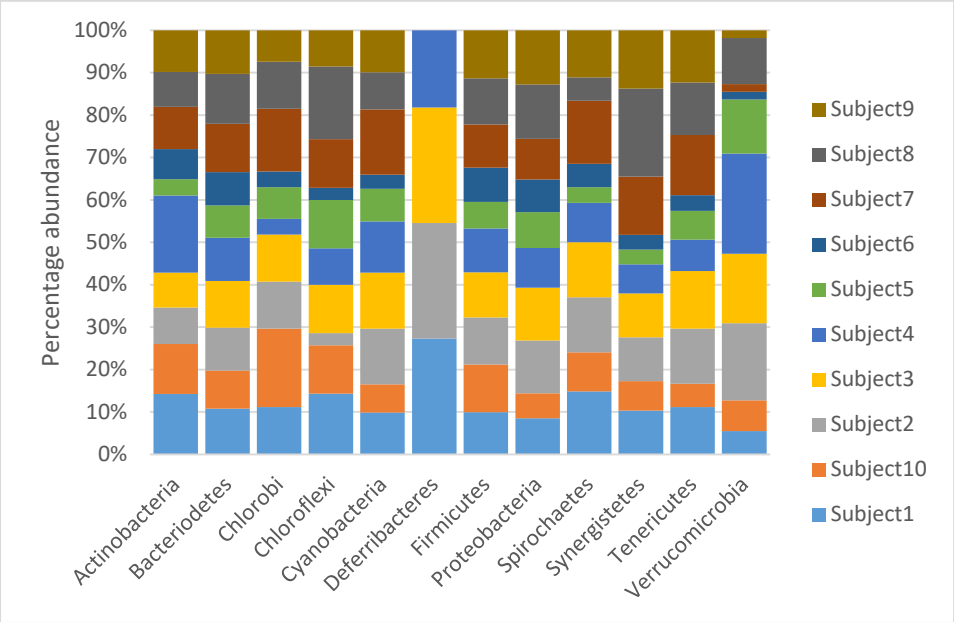

Fig. 1. Relative abundance of the Phyla taxa represented as 100% stack bar

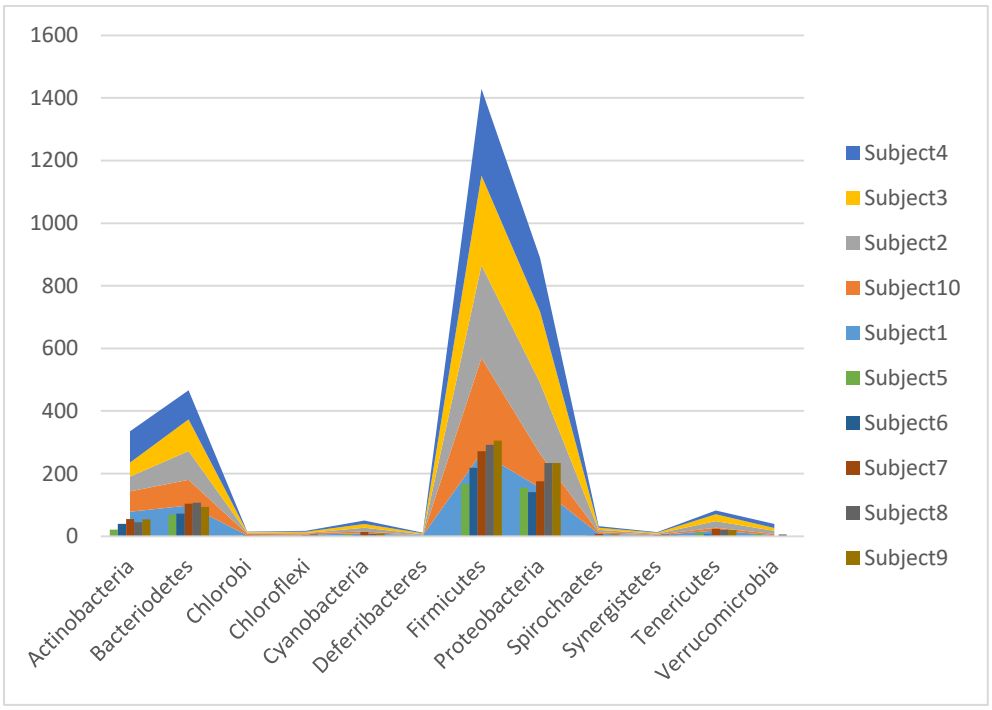

Fig. 2. Plot represents the abundance of phyla

Table 1. Correlation between relative abundance phyla and body mass index (BMI)

Phyla	Pearson correlation(r)	p-value
Firmicutes	0.363	0.127
Bacteriodetes	0.666	0.002*
Proteobacteria	0.362	0.045*
Actinobacteria	-0.292	0.225

**p value <0.05 was considered as significant*

Table 2. Correlation between relative abundance phyla and glycated hemoglobin

Phyla	Pearson correlation(r)	p-value
Firmicutes	0.176	0.471
Bacteriodetes	-0.042	0.864
Proteobacteria	0.03	0.901
Actinobacteria	0.171	0.483

p value <0.05 was considered as significant

5. CONCLUSION

It is speculated that gut microbiota associated with individual life style of obese T2DM subjects in the Nigerian population. The significant difference in gut microbiota could reflect to the distinct lifestyle and African dietary habits (high carbohydrate and fat intake, low fiber intake) and unregulated drug consumption. These Gram negative bacteria represented an intestinal microbiota signature associated with obese related T2DM individuals. Furthermore, the balance of these gram negative bacteria could help for prognosis of obese related Type 2 diabetes mellitus.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical approval for this study was obtained from Nnamdi Azikiwe University Teaching Hospital Ethics Committee (NAUTHEC).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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