(RESEARCH ARTICLE)

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Correlation of gut-*gammaproteobacteria* with body mass index and glycated haemoglobin in type-2 diabetes mellitus individuals in Nigeria

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Abstract

The aim of the study is to correlate the gut-*grammaproteobacteria* with body mass index and glycated haemoglobin in Type-2 diabetes mellitus individuals.

Methods: The gut microbiota signature of 110 adults was studied using 16S rRNA sequencing targeting V3–V4 hypervariable regions and obtained data using Pearson correlation analyzed with Statistical Package for the Social Science (SPSS version 26).

Results: It was observed that the relative abundance of Class *Bacteroidia* and *Gammaproteobacteria* were positively correlated with Body mass index as follows: *Bacteroidia* versus BMI(p>0.005); *Gammaproteobacteria* versus BMI (r=0.464,p=0.045*), whereas that of *Clostridia* versus BMI(p>0.005); *Actinobacteria* versus BMI (p>0.005); *Betaproteobacteri*a versus BMI (p>0.005); *Sphingobacteriia* versus BMI (p>0.005) and *Alphaproteinobacteria* versus BMI (p>0.005) demonstrated a negative correlation. This study also observed that the relative abundance of Clostridia versus Hb1Ac (p>0.005), and *Gammaproteobacteria* versus Hb1Ac (p>0.005) were positively correlated, whereas *Bacteroidia* versus Hb1Ac (p>0.005); *Actinobacteria* versus HbA1C (p>0.005); *Betaproteobacteria* versus Hb1Ac (p>0.005); Sphingobacteriia versus Hb1Ac (p>0.005) and *Alphaproteoobacteria* (p>0.005) demonstrated a negative correlation.

Conclusion: These results suggested that gut-*gammaproteobacteria* significantly increased in obese subjects. The highest trend was correlated with Type-2 diabetes related obese subjects. The low microbial diversity and depletion of some of specific taxa predisposed this T2D cohorts to gut dysbiosis.

Keywords: G*ammaproteobacteria*; *Clostridia*; *Betaproteobacteria*; *Alphaproteobacteria*; Type-2 diabetes; Obese

1. Introduction

Gammaproteobacteria is the most abundant class within the phylum *Proteobacteria* in type-2 diabetes [1]. Although, *Firmicutes* is more enriched in genera (~250) than all bacterial phyla [2].G*ammaproteobacteria* includes *Escherichia coli*, well-known pathogens *Salmonella*, *Yersinia*, *Vibrio*, and *Pseudomonas*, together with other pathogen found in the more basal and less well-resolved positions in the phylogeny such as *Coxiella* and *Francisella*, and endosymbionts that

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can be required for survival of their insect hosts. They exist in the presence of oxygen and temperature including chemoautotrophism and photoautotrophism [3]. Their characteristic features like rods, curved rods, cocci, spirilla, and filaments, and the class are common among the largest known bacterial cells [4]. Despite of the morphological features, 16S rRNA sequence relationship is now a golden method to define the class [3]. Metagenomic studies reveal that there is change in the relative proportions of gut microbial populations which could attribute to weight gain and insulin resistance, including alterations in *Gammaproteobacteria* and *Verrucomicrobia* and the ratios of *Firmicutes* to *Bacteroidetes* in type2 diabetes mellitus and possible alterations in butyrate-producing bacteria such as *Faecalibacterium prausnitzii* in diabetes mellitus [1]. Recent study showed that Lipopolysaccharides (LPSs) can induce metabolic endotoxemia, that is regarded as inflammatory and metabolically detrimental based on Toll-like receptor (TLR) 4 agonists, such as *Escherichia coli*-derived LPS. LPSs from certain bacteria antagonize [5].TLR4 yet attributed to endotoxemia measured by endotoxin units (EUs [5].From their findings, EUs reveals that only *E. coli* LPS promotes dysglycemia and adipose inflammation, delays intestinal glucose absorption, and augments insulin and glucagon-like peptide (GLP)-1 secretion [5]. However, the aim of the study is to correlate the gut-*grammaproteobacteria* with body mass index and glycated haemoglobin in Type-2 diabetes mellitus individuals.

2. 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.

2.1. Inclusion Criteria

Subjects for the study include male and female subjects between the ages 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.

2.2. Exclusive Criteria

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

2.3. 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.

2.4. 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.

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

2.5. 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.

2.6. 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.

2.7. Procedure Outline

2.7.1. Hemolysate Preparation

A 500μl 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.

2.7.2. 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.

2.7.3. DNA Extraction, PCR amplification, and sequencing

DNA was extracted individually from all subjects' stool samples using QiaAMP mini stool kit (Qiagen, Valencia, CA, USA) [6]. 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 (Flores *et al.,*2013). 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.

2.8. 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.

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3. Results

In subject 1 individual, the taxonomic representation at class level shows that Clostridia (31.14%), were the most abundant in the gut of subject 1 individual followed by *Bacterioidia* (11.17%), *Gammaproteobacteria* (11.54%), *Actinobacteria* (10.62%), Alphaproteobacteria (8.06%), *Deltaproteobacteria* (3.11%). Only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.

In subject 2 individual, the taxonomic representation at class level shows that Clostridia (28.85%), were the most abundant in the gut of subject 2 individual followed by Bacilli (14.32%), *Actinobacteria* (12.61%), *Bacterioidia* (11.54%), *Gammaproteobacteria* (8.55%), *Betaproteobacteria* (4.06%), *Sphingobacteria* (3.85%), *Deltaproteobacteria* (3.63%), *Alphaproteobacteria* (2.78%), *Erysipelotrichi*(2.14%), *Verrucomicrobiae* (1.50%). Only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.

In subject 3 individual, it was shown that Clostridia (28.76%), were the most abundant in the gut of subject 3 individual followed by *Gammaproteobacteria* (17.81%), *Bacilli* (14.32%), *Bacterioidia* (10.78%) *Actinobacteria* (12.61%), *Betaproteobacteria* (4.06%), *Sphingobacteria* (3.85%), *Deltaproteobacteria* (3.63%), *Alphaproteobacteria* (2.78%), *Erysipelotrichi*(2.14%), Verrucomicrobiae (1.50%). Only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.

In subject 4 individual, it was shown that Clostridia (28.74%), were the most abundant in the gut of subject 4 individual followed by *Gammaproteobacteria* (13.97%), *Bacilli* (13.77%), *Bacterioidia* (10.78%), *Betaproteobacteria* (9.18%), *Actinobacteria* (7.39%), Sphingobacteria (2.99%), *Alphaproteobacteria* (2.79%), Deltaproteobacteria (1.60%) Fusobacteria (1.00%), *Erysipelotrichi* (1.00%). Only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.

In subject 5 individual, it was observed that Clostridia (40.35%), were the most abundant in the gut of subject 4 individual followed by *Bacterioidia* (19.30%), *Gammaproteobacteria* (10.53%), *Bacilli* (7.46%), *Betaproteobacteria* (4.82%), *Sphingobacteria* (4.39%), *Deltaproteobacteria* (3.07%), *Methanobacteria* (1.75%), *Erysipelotrichi* (1.75%) *Actinobacteria* (1.32%), *Alphaproteobacteria* (1.32%), Synergista (1.32%). Only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.
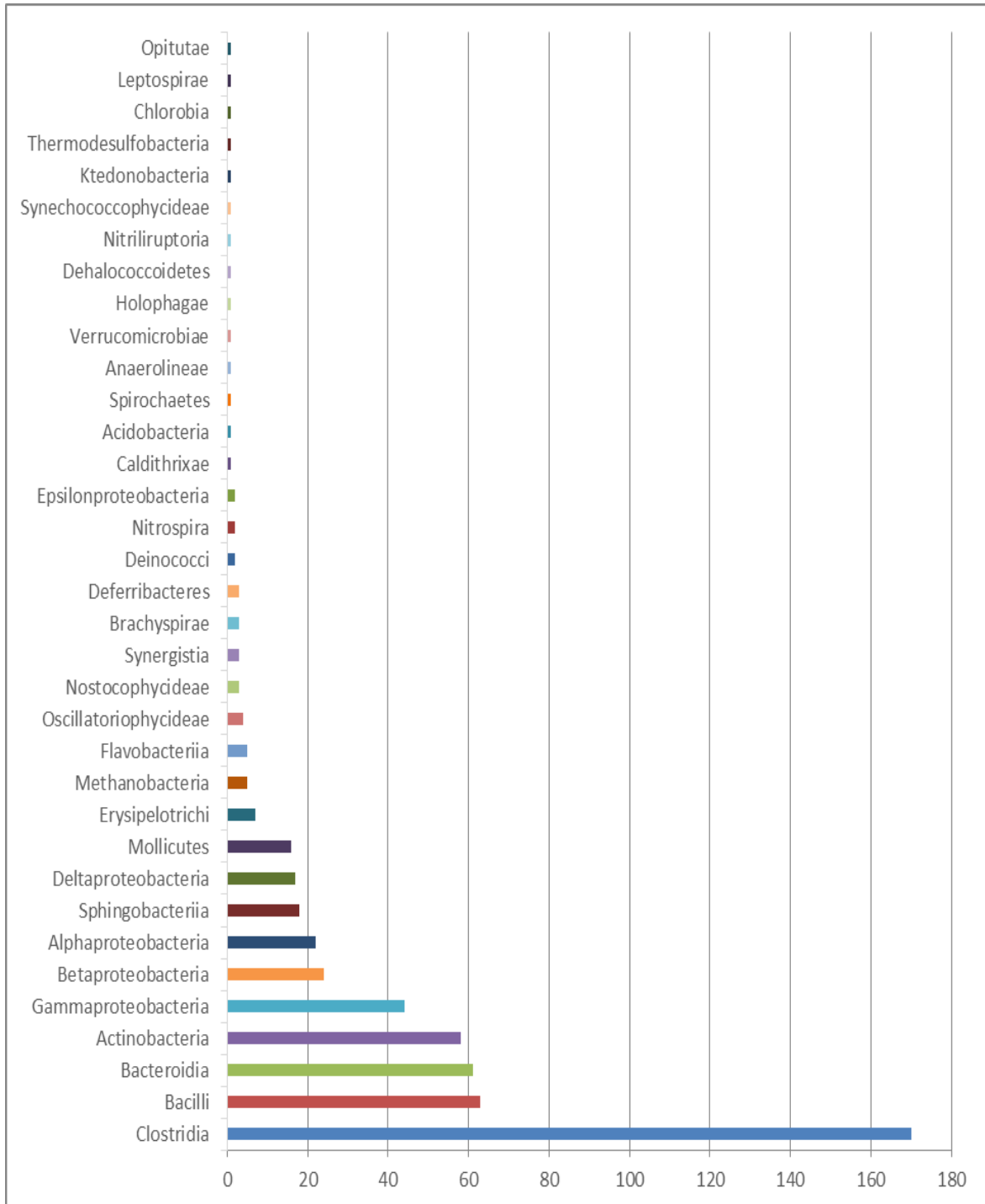

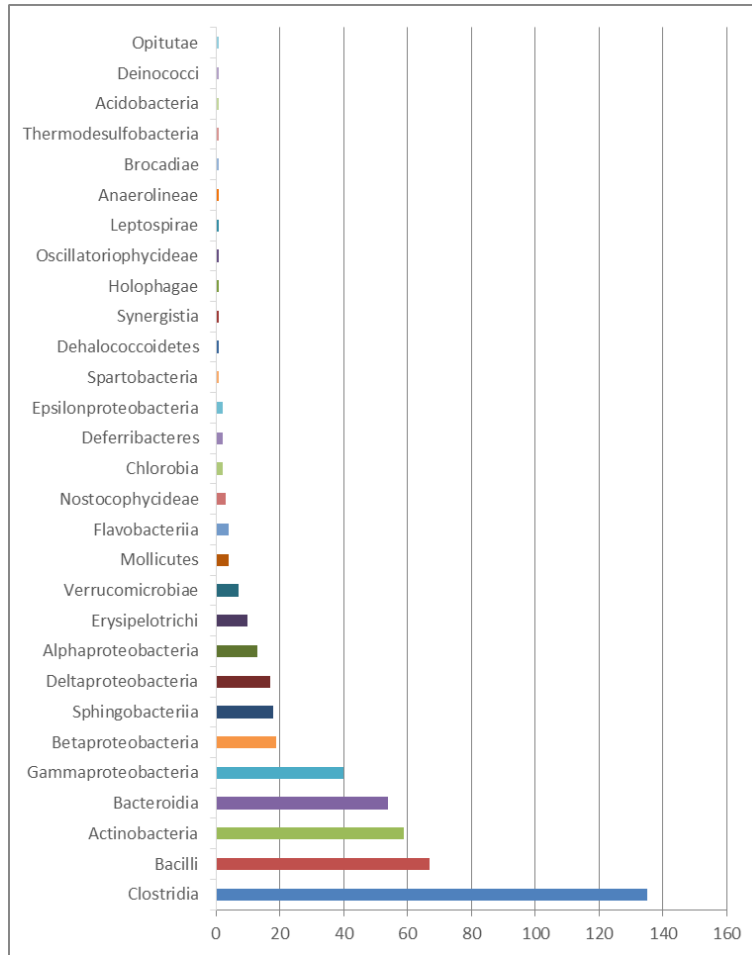**Figure 1** Subject One individual with obese related T2DM of Relative Abundance of classes

Figure 2 Subject Two individual with obese related T2DM of Relative Abundance of classes
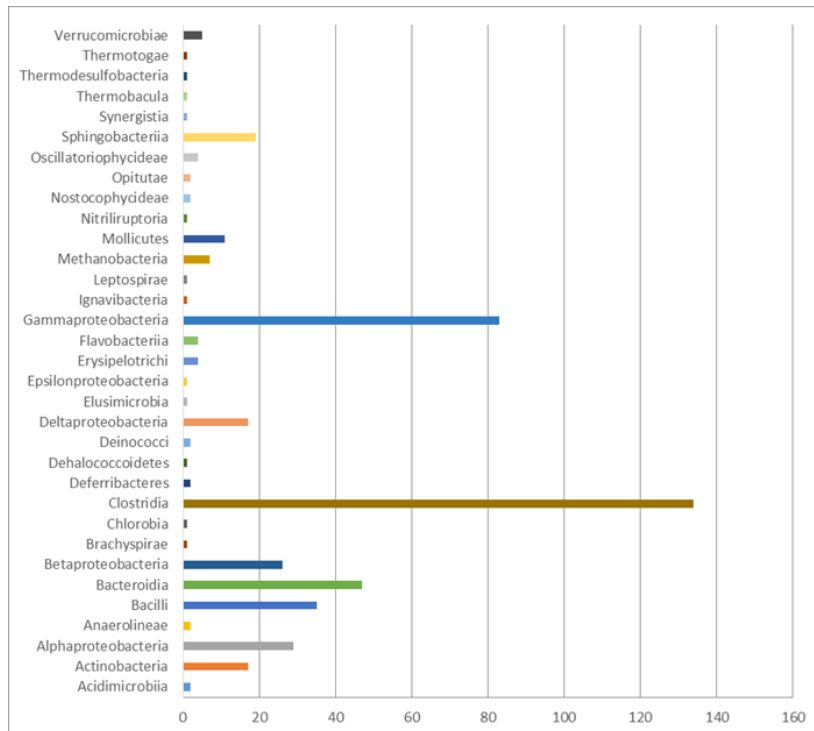

Figure 3 Subject Three individual with obese related T2DM of Relative Abundance of classes
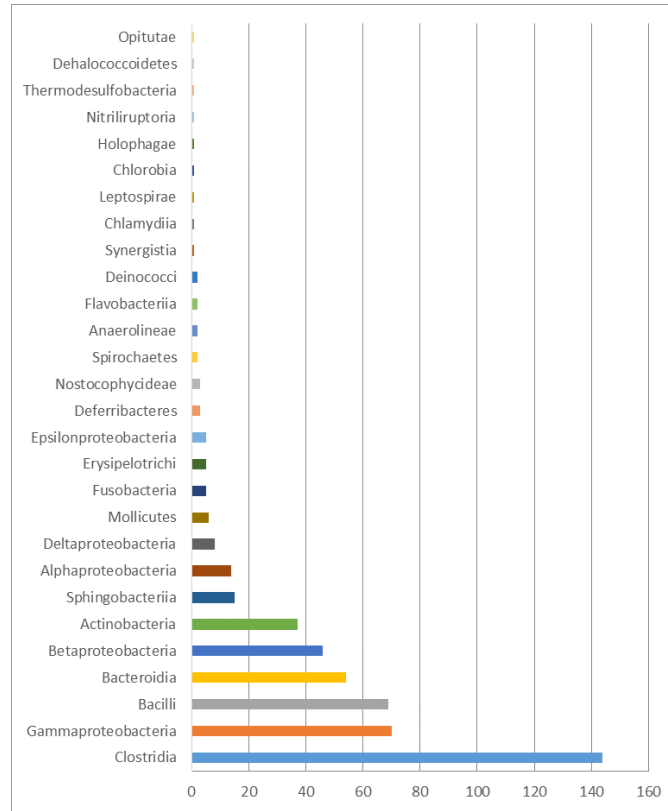
Figure 4 Subject Four individual with obese related T2DM of Relative Abundance of classes
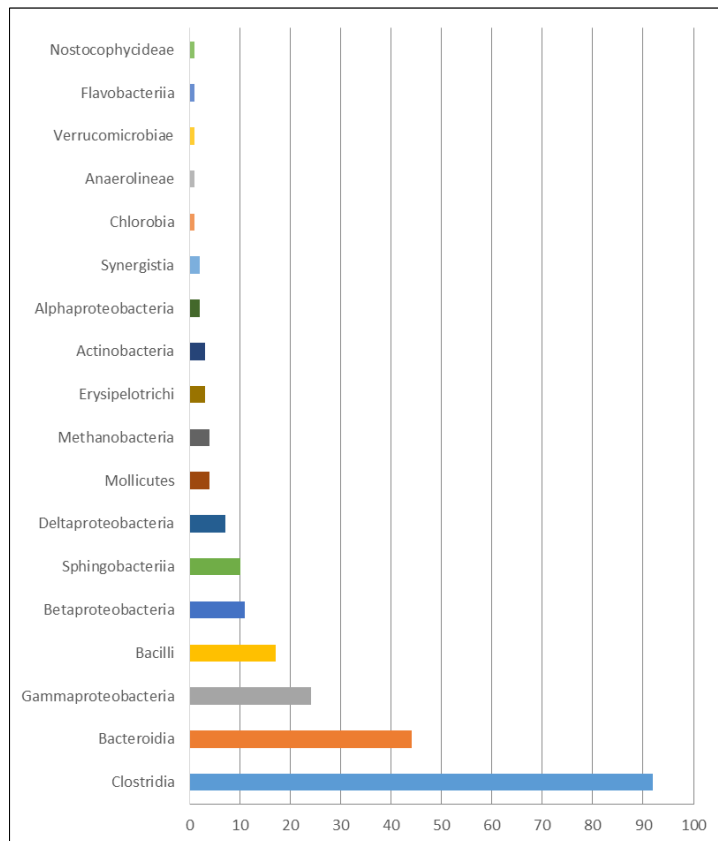

Figure 5 Subject Five individual with obese related T2DM of Relative Abundance of classes

It was observed that the relative abundance of Class *Bacteroidia* and *Gammaproteobacteria* were positively correlated with Body mass index as follows: *Bacteroidia* versus BMI(r = 0.295, p = 0.221); *Gammaproteobacteria* versus BMI (r=0.464,p=0.045*), whereas that of *Clostridia* versus BMI (r = -0.383, p = 0.105); *Actinobacteria* versus BMI (r = - 0.270, p = 0.264); *Betaproteobacteria versus* BMI (r = - 0.228, p = 0.348); *Sphingobacteriia* versus BMI (r = - 0.139, p = 0.570) and *Alphaproteinobacteria* versus BMI (r = - 0.248, p = 0.306) demonstrated a negative correlation. It was also shown that *Gammaproteobacteria* was significantly positive with BMI (Table1). This study also observed that the relative abundance of *Clostridia* versus Hb1AC(r = 0.154, p = 0.530), and *Gammaproteobacteria* versus Hb1AC (r = 0.109, p = 0.656) were positively correlated with Glycermic control , whereas that of *Bacteroidia* versus Hb1AC (r = -0.149, p = 0.542); *Actinobacteria* versus HbA1C (r = - 0.065, p = 0.792); *Betaproteobacteria* versus Hb1AC(r = - 0.297, p = 0.217); *Sphingobacteriia* versus Hb1AC (r = - 0.107, p = 0.570) and *Alphaproteinobacteria* (r = - 0.132, p = 0.589) demonstrated a negative correlation(Table 2).

Table 1 Correlation between relative abundance of classes and Body mass index (BMI)

Classes	Pearson correlation(r)	p-value
Clostridia	-0.383	0.105
Bacteroidia	0.295	0.221
Actinobacteria	-0.270	0.264
Betaproteobacteria	-0.228	0.348
Sphingobacteriia	-0.139	0.570
Alphaproteobacteria	-0.248	0.306
Gammaproteobacteria	0.464	0.045*

*p value <0.05 was considered as significant; only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.

Table 2 Correlation between Classes of relative abundance and Glycated heamoglobin

Classes	Pearson correlation(r)	p-value
Clostridia	0.154	0.530
Bacteroidia	-0.149	0.542
Actinobacteria	-0.065	0.792
Betaproteobacteria	-0.297	0.217
Sphingobacteriia	-0.107	0.663
Alphaproteobacteria	0.132	0.589
Gammaproteobacteria	0.109	0.656

P-value <0.05 was considered as significant; Only the taxonomy of class with relative abundance above 1% in obese related type-2 diabetes mellitus individuals was listed.

4. Discussion

Gammaproteobacteria belong to the class of pathogenic bacteria and were found to be greater in abundant in Obese related type-2 diabetes mellitus [7]. This findings showed that *Gammaproteobacteria* were the second and or third class abundance among obese- related type-2 diabetes mellitus individual after class-Clostridia.

It was observed that the relative abundance of Class *Bacteroidia* and *Gammaproteobacteria* were positively correlated with Body mass index, whereas that of *Clostridia* versus BMI; *Actinobacteria* versus BMI; *Betaproteobacteria versus* BMI; *Sphingobacteriia* versus BMI and *Alphaproteobacteria* versus BMI demonstrated a negative correlation. It was also shown that *Gammaproteobacteria* was significantly positive with BMI.

This study also observed that the relative abundance of *Clostridia* versus Hb1Ac, and *Gammaproteobacteria* versus Hb1Ac were positively correlated with Glycated heamoglobin, whereas that of *Bacteroidia* versus Hb1Ac; *Actinobacteria* versus HbA1c; *Betaproteobacteria* versus Hb1Ac; *Sphingobacteriia* versus Hb1Ac and *Alphaproteobacteria* demonstrated a negative correlation. This study observed that the relative abundance of order *Gammaproteobacteria* was significantly positive correlated with obese related type 2 diabetes mellitus. This finding is in contrast to previous study carried out by Ahmad *et al.*, [8] that reported significantly reduced abundance of *Gammaproteobacteria* in obese-T2DM subjects. Though, these bacteria are extremely important for their contribution toward the development of disease as their increased abundance might have elevated the lipopolysaccharide (LPS) level in obese-T2DM individual. The circulating LPS binds with CD14 and mediates an inflammatory response, thus contributing positively to the development of obesity and insulin resistance [9; 10; 11; 12].

5. Conclusion

These results suggested that gut-*gammaproteobacteria* significantly increased in obese subjects. The highest trend was correlated with Type-2 diabetes related obese subjects. The low microbial diversity and depletion of some of specific taxa predisposed this T2D cohorts to gut dysbiosis.

Compliance with ethical standards

Disclosure of conflict of interest

The author declared that there is no conflict of interest

Statement of ethical approval

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

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