



## Research article

## Insights into the gut microbiota of Nigerian elderly with type 2 diabetes and non-diabetic elderly persons

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## ARTICLE INFO

## Keywords:

Microbiology  
Ecology  
Bioinformatics  
Genetics  
Health sciences  
Type 2 diabetes  
Elderly  
Gut microbiota  
Nigeria

## ABSTRACT

Type 2 diabetes (T2D) is a prevalent non-communicable disease among the world's growing elderly population. The contribution of the gut microbiota to T2D in several Westernized countries has been established. However, there is little information on the role of the gut microbiota in T2D from the African continent where lifestyle and life expectancy are different.

**Aims:** This study sought to investigate gut microbiota variation in relation to elderly people living with T2D in Nigeria.

**Methods:** Whole microbial community DNA were derived from the stool samples of healthy urban-dwelling elderly individuals and urban-dwelling elderly individuals with T2D. The V4 region of the 16S rRNA gene was Illumina-sequenced and analyzed using QIIME2.

**Results:** Beta taxonomic diversity was significantly different between healthy elderly individuals and elderly individuals with T2D. However, no difference in the alpha taxonomic diversity and predicted functional alpha diversity of the gut microbiota was observed. The genus *Ruminococcus* (T2D versus Healthy: 2.89% vs 2.21%), families *Coriobacteriaceae* (*Collinsella*, T2D versus Healthy: 2.62 % vs 1.25%) and *Bifidobacteriaceae* were enriched in elderly individuals with T2D, while members of *Clostridiaceae* (*Clostridium*, Healthy versus T2D: 5.6% vs 3.2%) and *Peptostreptococcaceae* (Healthy versus T2D: 3.45% vs 1.99%) were enriched in healthy volunteers. Pathways involved in amino acid biosynthesis were enriched in elderly individuals with T2D, while pathways involved in respiration and the biosynthesis of vital building blocks were enriched in healthy volunteers.

**Conclusions:** The study demonstrated for the first time in an African elderly population that the abundance of *Bifidobacteriaceae*, *Collinsella*, and *Ruminococcus* within the gut varies in relation to T2D. Findings from this study suggest that the restoration of features associated with healthiness via the way of gut microbiota modification could be one step needed to improve elderly patient care.

## 1. Introduction

Type 2 diabetes (T2D), the most prevalent endocrine disease worldwide [1], is characterized by long-term high blood sugar levels resulting from the body's inability to make use of insulin produced [2]. Currently, T2D prevalence has reached epidemic proportions globally [3]. It is projected that T2D will be the seventh leading cause of death by the year 2030 [4], and that 700 million individuals in the world will be living with diabetes by 2045 [2]. One of the contributing factors to the increasing prevalence of T2D is aging, which in turn, is a consequence of increased global life expectancy [5]. Diabetes is an important health challenge in the elderly as the global prevalence among the elderly is

currently greater than that of children and adults below the age of 60 [6]. Currently, 20% of the elderly population around the world are living with diabetes [2]. Many of the co-morbidities associated with diabetes are of great negative consequences to the elderly population. Such co-morbidities include; nephropathy, neuropathy, retinopathy, macular oedema resulting in blindness, diabetic-related amputations, end-stage renal disease, strokes, and acute myocardial infarctions [5]. The countries with the highest incidence of diabetes in the world include; China, India, and the United States of America [2].

Nigeria is the most populous nation in sub-Saharan Africa with a projected estimated population of over 200 million people as at the year 2018 ([https://www.indexmundi.com/nigeria/demographics\\_profile](https://www.indexmundi.com/nigeria/demographics_profile)

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Received 14 February 2020; Received in revised form 9 April 2020; Accepted 7 May 2020

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.html). In Nigeria, the number and proportion of people aged 60 years and above has increased from below 1% in the mid-2000s to 3.26% in 2018 ([https://www.indexmundi.com/nigeria/demographics\\_profile.html](https://www.indexmundi.com/nigeria/demographics_profile.html)). Although there is limited epidemiological evidence on the incidence and prevalence of T2D in Nigeria, available estimates suggest that about 8–10% of the population are affected, numbering about 10 million people [2, 7]. Of these, 6–9% are among the elderly [8] which constitutes the largest number of the elderly living with T2D in sub-Saharan Africa. This once-considered rare non-communicable disease in Sub-Saharan Africa is now endemic, affecting both young adults and the elderly alike and is likely due in part, to increased life expectancy, urbanization, incorporation of western dietary habits and sedentary lifestyle [9].

Although T2D is thought to be associated with numerous factors, advances in next-generation sequencing has opened our eyes to yet another contributor to this epidemic—the gut microbiota. The human gastrointestinal tract is colonized by a vast number of microorganisms, forming a gut microbial community that play a huge role in its host's health. Numerous pieces of evidence suggest that the gut microbiota is associated with T2D [10–13]. One of the first published studies on the variation in the gut microbiota of European individuals with T2D revealed a depletion in the abundance of members of the dominant phylum Firmicutes in individuals living with T2D when compared with the healthy population [14]. Another study on an Asian population reported the use of butyrate producers and members of the Verrucomicrobia as potential biomarkers of T2D [15]. Likewise, a study on an African-American population reported that the abundance of *Bifidobacterium* and *Prevotella* varies with T2D, opioid use, and the drug metformin [16]. Although studies have shown an association of gut microbiota changes with diabetes in Asians [1, 3, 15, 17–19], Europeans [12–14, 17], North American population [16], and African young population [20], these studies focus on human populations with wide age ranges (especially adults), inadvertently revealing insufficient information on the gut microbiota changes with T2D of the growing elderly population. Furthermore, less is known and understood about the features associated specifically with the gut microbiota of African elderly living with diabetes.

Efforts to define extents of disturbances in the communities of the gut microbiota of elderly diabetic subjects from Nigeria will help us to understand the association between gut microbiota compositional changes and diabetes in a subset of the Sub-Saharan African populace. Furthermore, the detection of microbial features/biomarkers associated with diabetes is key to understanding the potential role they play in the diabetic state. Therefore, the objective of this study was to characterize the structure and predicted function of the gut microbiota of elderly individuals with type 2 diabetes as compared to healthy urban elderly population residing within the same geographical location, and assess whether there are differences in the diversity of the gut microbiota in elderly individuals living with diabetes when compared with healthy elderly volunteers.

## 2. Results

### 2.1. Physical, demographics and clinical data

In this study, we examined the composition and predicted functions of the faecal microbiota in a population of elderly individuals (above 60 years of age), comprising the elderly who have diabetes ( $n = 20$ , Male = 6, Female = 14) and healthy urban elderly volunteers ( $n = 22$ ; Male = 14, Female = 8). Both study populations reside in the same geographical location, are exposed to the same climate. The healthy urban elderly and elderly subjects with Type 2 diabetes consume slightly similar diets, including fruits and vegetables, with the exception of the exclusion of tuber and cassava-based products as well as animal protein (including dairy products) from the diet of the diabetic individuals. Furthermore, elderly individuals who have diabetes have modified their diets according to the doctor's prescriptions in order to manage T2D effectively.

They consume plantain (boiled unripe plantain) and its products (such as plantain flour), and special types of local rice. They also add a large dose of vegetables in their meals including Fluted pumpkin (*Ugwu*), Bitter leaves (*Ewuro*), garden egg and cucumber. Further information on the diet of healthy urban elderly population and elderly living with T2D are presented in Table 1.

More than half of the healthy urban elderly individuals were of Yoruba ethnic group, had retired from active service, married, and had attained the tertiary educational level (that is, university education (BSc, MSc, or PhD) or Nigerian certificate in Education (NCE)) (Table 2). Those in active service were traders (19%), artisans (9%), traditional medical practitioners (4%), contractors (4%), or gatemen (4%). On the other hand, the majority of the diabetic elderly individuals were female, of Yoruba ethnic group, were either married or widowed, and had attained at least the primary level of education. Many of diabetic elderly volunteers were retired (55%), while the rest (45%) were still in active service (20% of them were traders, 10% were Clergymen, 10% were businessmen, while 5% were legal practitioners) (Table 2).

The urban elderly individuals were healthy individuals who, often use analgesics (paracetamol, panadol) and blood tonic, because old age is associated with frailty which is manifested in body and joint pain. Elderly individuals who have diabetes were administered Metformin (the drug of choice for individuals having diabetes), analgesics, vitamins and mineral supplements, and antihypertensive drugs (for the elderly having hypertension together with diabetes).

### 2.2. Similarities and differences between the taxonomic profiles of the gut microbiota of diabetic and healthy urban elderly individuals

We characterized the gut microbiome of 22 healthy urban-dwelling elderly volunteers and 20 urban-dwelling elderly volunteers who have diabetes and undergo medical checkup once every quarter at the University College Hospital, Ibadan, Nigeria. Following Illumina sequencing, a total of 3,491,662 high-throughput sequence data with a per sample mean of  $83,134 \pm 22,590$  reads were generated. Following the completion of quality filtering steps and rarefaction of sample reads to 10,000, a total of 4,090 features were included for downstream analysis. No significant difference in the taxonomic and functional alpha diversity was observed using metrics such as Faith's phylogenetic diversity ( $p = 0.07$ ), evenness index ( $p = 0.18$ ), observed OTUs ( $p = 0.49$ ), and Shannon index ( $p = 0.28$ ) (Figure 1a-d).

Interestingly, significant distinctions in the taxonomic beta diversity of the gut microbiota in both study groups were observed with Unweighted Unifrac distance metric (PERMANOVA,  $p = 0.012$ ; (Adonis,  $p = 0.019$ ,  $R^2 = 0.04$ ); (ANOSIM,  $p = 0.024$ ,  $R = 0.083$ )) and Jaccard distance metric (PERMANOVA,  $p = 0.016$ ; (Adonis,  $p = 0.022$ ,  $R^2 = 0.03$ ); (ANOSIM,  $p = 0.005$ ,  $R = 0.11$ )) (Figure 2a). However, no significant distinctions in the functional beta diversity of the gut microbiota was observed in the healthy urban elderly individuals and elderly individuals who have T2D (Figure 2b).

At the phylum level, a total of 22 phyla were observed in the study groups, including the dominant phyla Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria, with Firmicutes (~72%) being the most abundant phyla in both study groups, followed by Proteobacteria (~11%) (Figure 3a-b). At the level of the family, *Lachnospiraceae*, *Ruminococcaceae*, and *Enterobacteriaceae* were the most dominant in both study groups, with *Lachnospiraceae* (~30%) being the most abundant family, followed by *Ruminococcaceae* (~19.5%) (Figure 3c-d). At the genus level, the most dominant are members of the *Ruminococcaceae* (unknown genus, ~ 8%) and *Lachnospiraceae* (*Blautia*, ~ 7%) family (Figure 3e-f).

Taxonomic biomarkers associated with elderly individuals who have diabetes include members of Actinobacteria phylum (T2D versus Healthy: 3.35% vs 1.53%), *Coriobacteriaceae* family (T2D versus Healthy: 2.62% vs 1.25 %) and the genera *Ruminococcus* and *Collinsella*. On the other hand, members of *Peptostreptococcaceae* (Healthy versus T2D:

**Table 1.** Dietary habits of healthy urban and diabetic elderly.

Food Type	Healthy Urban Elderly	Elderly who have diabetes
1 Grains	Oats, wheat, millet, sorghum, local and refined rice	Local rice
2 Fruits	Orange, Banana	Cucumber
3 Vegetables	Ugwu/fluted pumpkin, African Spinach ( <i>Efo tete</i> )	Ugwu/fluted pumpkin, bitter leaves, garden egg
4 Protein	Beans, bean product ( <i>Akara, Moin-moin</i> ), animal proteins (meat, fish, egg, milk).	Animal protein not consumed.
5 Tuber and tuber products	Cooked Yam, Pounded yam, Cooked Cocoyam, <i>Amala</i>	Not consumed
6 Cassava products	<i>Fufu, Eba, Gaari</i> , Bread	Not consumed
7 Dairy and dairy products	Milk, eggs	Not consumed
7 Other foods high in carbohydrate	Semovita and Semolina, corn and corn products ( <i>Ogi, Eko</i> ), processed food (Indomie).	Boiled unripe plantain, plantain flour

3.45% vs 1.99%) and *Clostridiaceae* families (including *Clostridium*, Healthy versus T2D: 5.6% vs 3.2%) were associated with the healthy urban elderly population (Figure 4a). On the one hand, functional pathways involved in the biosynthesis of GDP-mannose-derived O antigen building blocks and colonic acid building blocks, GDP mannose, and isopropanol were some of the pathways associated with the healthy urban elderly population. Furthermore, pathways involved in photorespiration, urea cycle, aerobic respiration (cytochrome c) and the degradation of glycerol to butanol were enriched in the healthy urban elderly population. On the other hand, pathways involved in the biosynthesis of S-adenosyl-L-methionine and L-lysine were predicted to be associated with elderly individuals who have diabetes (Figure 4b).

### 3. Discussion

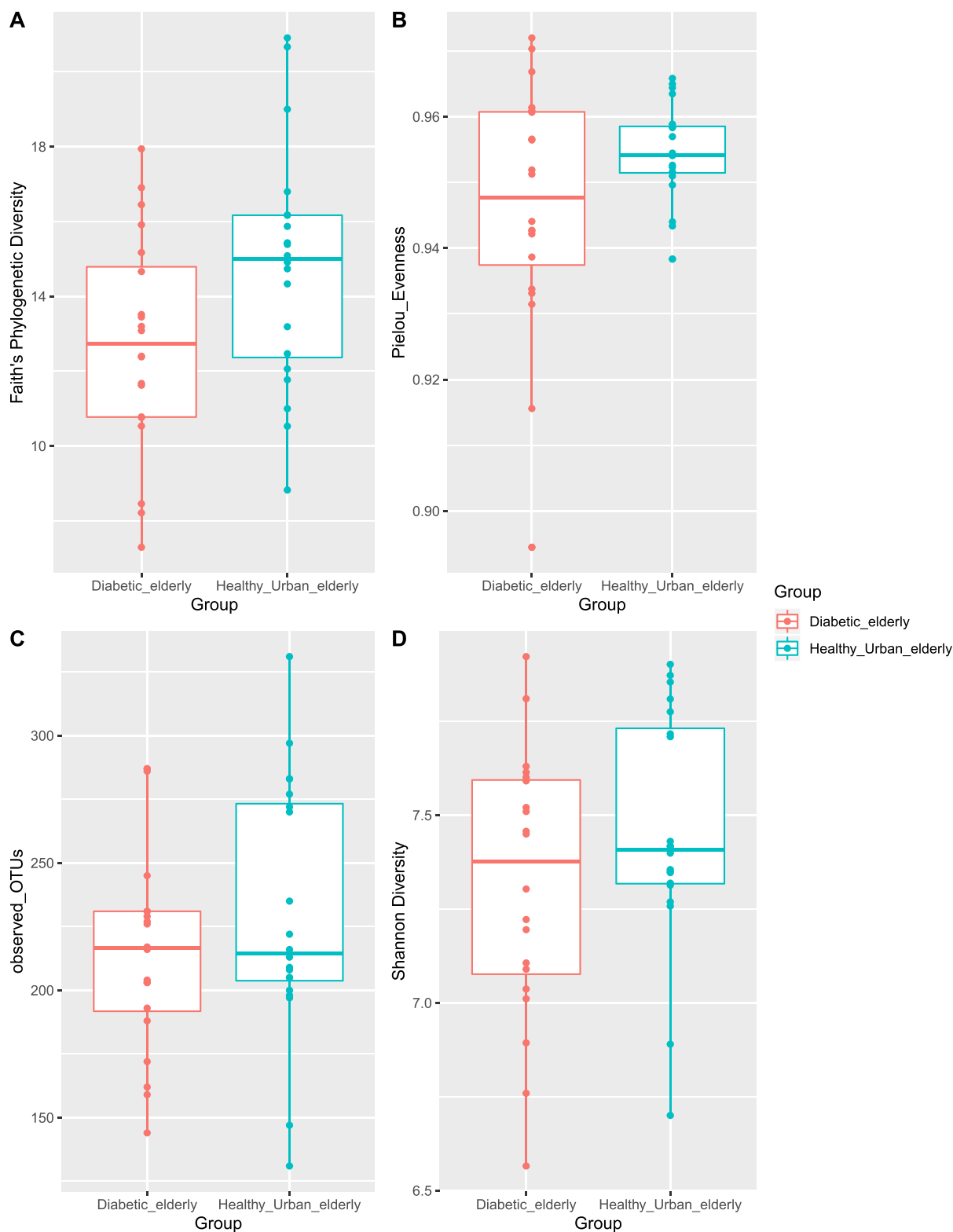
In this study, we compared the gut microbiota of healthy elderly individuals and elderly individuals who have T2D in Ibadan, Nigeria, for the purpose of understanding better the differences in the gut microbiota of the two study populations and the features associated with T2D in elderly individuals ( $\geq 60$  years). Based on their diet, lifestyle, geographical location, ethnicity, occupation, and age, the study groups could be considered as a relatively homogenous cohort.

Our study revealed that the dominant microbial phyla in the gut of the study participants include; Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria phyla, supporting reports from previous studies [21, 22] on the human population. Furthermore, the abundance of Proteobacteria in the elderly has been associated with dysbiosis in the gut microbiota, because members of this phylum are opportunistic pathogens which under favourable conditions, increase the risk of disease in humans, especially the elderly who have an impaired immune system functionality [23]. The prevalence of Proteobacteria in the elderly is likely enhanced further by age-related physiological changes (including; reduction in intestinal motility, increased intestinal permeability), and decline in the function of the gut-associated immune system typified by increased low-grade systemic inflammation [21].

We demonstrated from our research that there are differences in the gut microbiota of healthy elderly volunteers and elderly volunteers living with T2D. members of phylum Actinobacteria (families *Bifidobacteriaceae* and *Coriobacteriaceae* (*Collinsella*)), and genus *Ruminococcus* (*Ruminococcaceae* family) were enriched in elderly individuals who have diabetes. It is surprising to observe the enrichment of members of the family *Bifidobacteriaceae* in elderly individuals having diabetes in our study, as it contrasts previous studies reporting the enrichment of family *Bifidobacteriaceae* in healthy individuals rather than in T2D individuals

**Table 2.** Sociodemographic characteristics of the elderly volunteers.

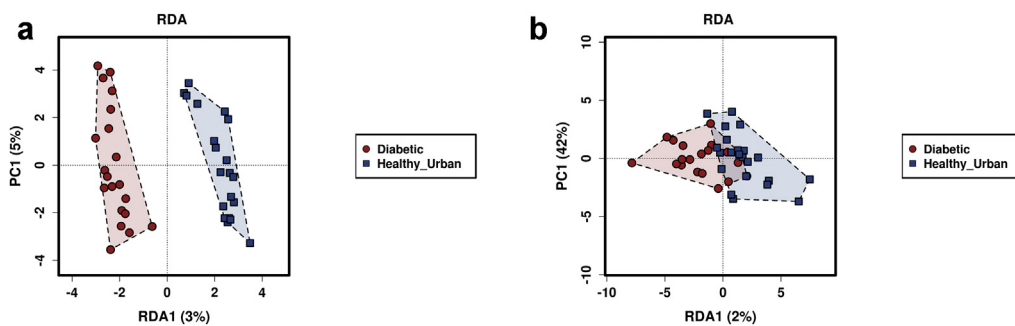
Variable	Urban elderly (n = 22)	Diabetic Elderly (n = 20)	P -value
<b>Age (in years)</b>	60–80	61–81	
60–70	11	12	
71–80	10	8	
>80	0	1	
<i>Chi-square Test</i>			0.89
<b>Mean age (years)</b>	68.67 ± 6.04	70.35 ± 6.45	
<i>Mann-Whitney U test</i>			0.32
<b>Gender</b>			
Male (%)	14 (63.6)	6 (30.0)	
Female (%)	8 (36.4)	14 (70.0)	
<i>Chi-square Test</i>			0.03
<b>Ethnicity</b>			
Yoruba (%)	21 (95)	17 (85)	
Ibo (%)	1 (5)	2 (10)	
Others (%)	0 (0)	1 (5)	
<i>Chi-square Test</i>			0.76
<b>Occupation</b>			
Retired (%)	13 (57)	11 (55)	
In Active service (%)	9 (43)	9 (45)	
<i>Chi-square Test</i>			0.79
<b>Level of Education</b>			
Primary (%)	4 (19)	12 (60)	
Secondary (%)	6 (29)	4 (20)	
Tertiary (%)	12 (52)	4 (20)	
<i>Chi-square Test</i>			0.01



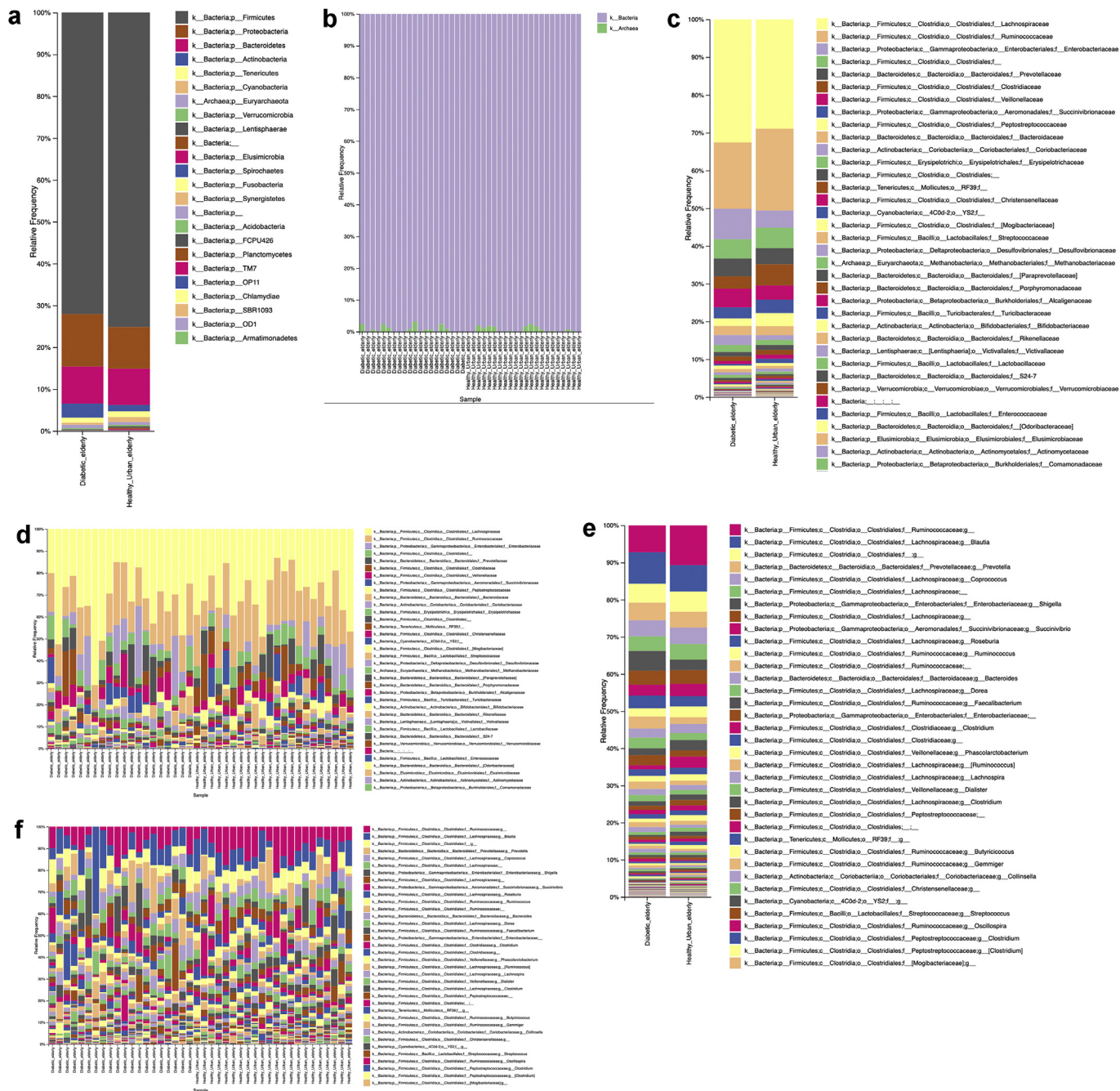
**Figure 1.** Differences in parameters of alpha diversity of the gut microbiota in healthy elderly volunteers and elderly volunteers with diabetes. Boxplots show no significant difference in the Faith's phylogenetic index (a), evenness index (b), observed OTUs (c), and Shannon index (d) in the gut microbiota of healthy elderly volunteers and elderly volunteers with diabetes. Mann-Whitney U test was used with p-values of 0.07 (a), 0.18 (b), 0.49 (c), and 0.28 (d).

[13, 19, 24]. Since members of the *Bifidobacteriaceae* have been associated with improved glucose tolerance [25], the enrichment of *Bifidobacteriaceae* in elderly individuals living with diabetes could signal improved glycemic control, which could result from physician-prescribed changes in dietary habits. Also, it is worthy to note

that in previous studies on some Nigerian populations [26, 27], members of the family *Bifidobacteriaceae* seem to have been depleted in healthy individuals, which suggests a country-specific deviation from the norm. In contrast to our study, there was a negative correlation of *Coriobacteriaceae* with diabetes, based on the reports from previous

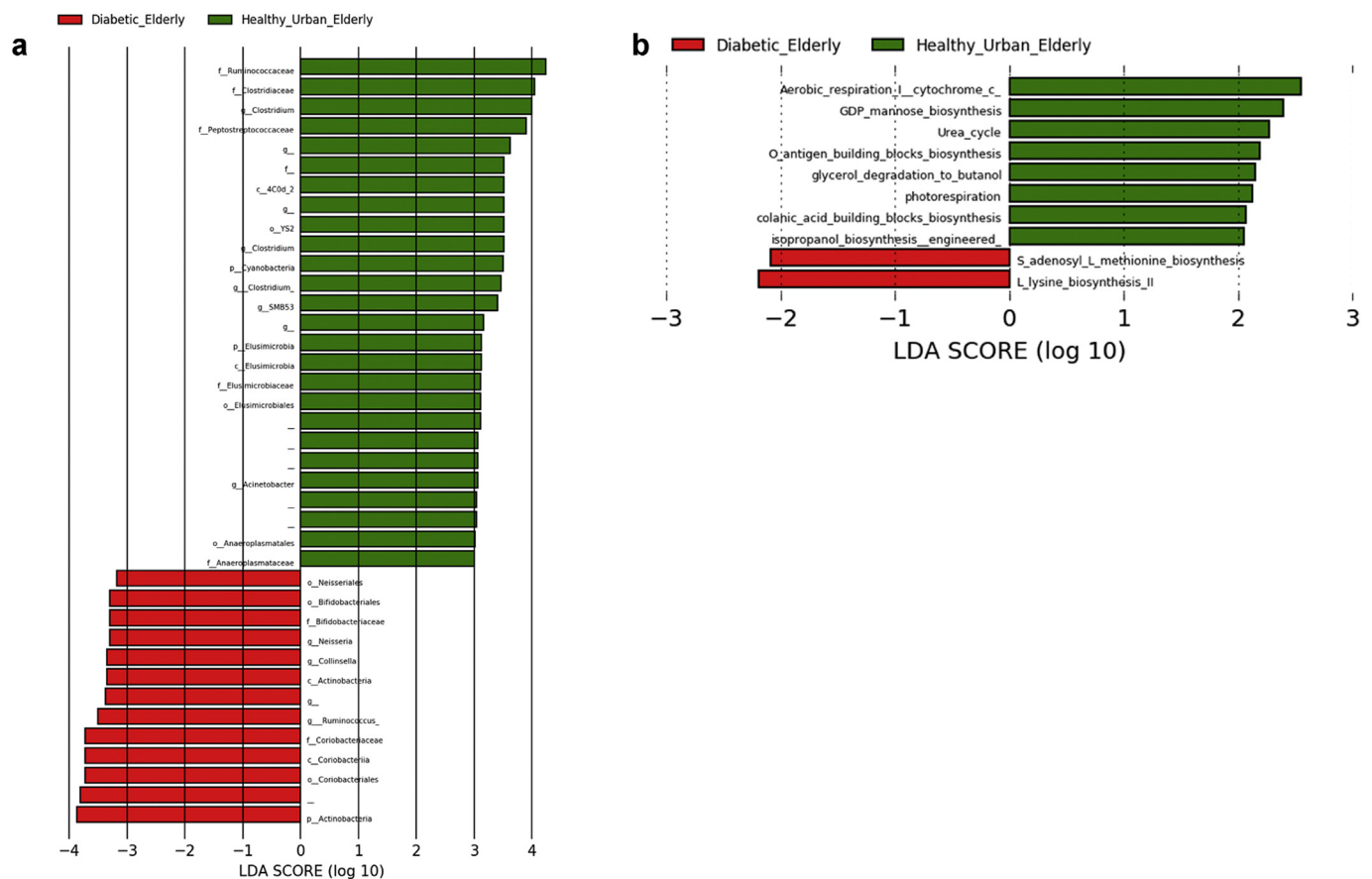


**Figure 2.** Differences in parameters of taxonomic beta diversity of the gut microbiota in healthy elderly volunteers and elderly volunteers with diabetes. RDA plots show a significant difference in gut microbial community composition (a) with Unweighted Unifrac distances and Jaccard distances (PERMANOVA test was used with p-values of 0.012 and 0.016, respectively), but show no significant distinctions in the functional gut microbiota composition (b).



**Figure 3.** Summary of gut microbial community taxa abundance in the healthy elderly and elderly with diabetes. Bar plots show taxa summary and individual taxonomic variation at the phylum level (a, b), family level (c,d), and genus level (e,f).





**Figure 4.** Differential abundance of discriminant features based on microbial taxa (a) and PICRUSt-predicted functional pathways (b) in the healthy elderly (green) and elderly who have diabetes (red).

studies [13, 19]. The difference in the association of *Coriobacteriaceae* with either the healthy state or the diabetic state could be due to inter-ethnic, intercountry, or intercontinental differences in the gut microbiota of study participants from different parts of the world. Interestingly, one member of the *Coriobacteriaceae* family, *Collinsella*, has been reported to be positively associated with type 2 diabetes and atherosclerosis [15, 28], thereby supporting our findings of the association of *Coriobacteriaceae* with T2D. Members of the family *Ruminococcaceae* are human gut commensals responsible for the catabolism of plant polysaccharides [29]. They have been suggested to be one of the microbial families to be used as faecal indicators of water pollution and contamination in previous reports due to their dominance in the gut of humans globally [30]. Compositional alteration of the members of the family *Ruminococcaceae* has been linked to high-fat diet [31] and the potential occurrence of metabolic diseases such as T2D [19].

On the other hand, members of the family *Clostridiaceae* (including *Clostridium*) and members of *Peptostreptococcaceae* were enriched in the gut of healthy urban elderly population. As this taxon is depleted in elderly individuals living with T2D, it appears that members of *Clostridiaceae* play a major role in the stability of the gut microbiota and the health of its human host. It is worthy to note that members of the *Clostridiaceae* and *Peptostreptococcaceae* families have been reported to be dominant in the gut of healthy Nigerians [26, 27]. Our finding is supported by Karlsson and colleagues who reported the low abundance of *Clostridium* in European women with T2D, and noted that there was a negative correlation of *Clostridium* with fasting blood sugar, glycated haemoglobin, insulin, and plasma triglycerides [13]. Furthermore, Wang reported the enrichment of *Clostridiaceae* and *Peptostreptococcaceae* in the gut of healthy urban individuals from the Kazak ethnic group [19]. Many of the members of the *Clostridiaceae* family are renowned for butyrate production from the fermentation of carbohydrates, which is of great

benefit to intestinal epithelial cells as butyrate serves as a source of energy, as well as a modulator of the immune system [32]. Members of the *Peptostreptococcaceae* assist in the maintenance of human and animal gut microbiota homeostasis [33]. It is a little wonder that these two families are enriched in the healthy elderly population, and could serve as potential microbial biomarkers of healthiness whose abundance can be increased in T2D elderly individuals, in order to restore the gut microbiota to the state of that of the healthy population.

No significant difference in the taxonomic and functional alpha diversity of the gut microbiota between the two study groups was observed. This observation is in agreement with Wu's study (35–65 years) [24], Larsen's study (males only; age range: 31–73 years) [14], and Zhang's study (age range: 52–54 years) [15], where they observed no significant difference in the species diversity (alpha diversity) between the diabetic group and the non-diabetic group. The lack of difference in the within-sample diversity could have resulted from the physician-prescribed dietary changes and medications (e.g. metformin) observed by individuals living with T2D that ultimately led to the improvement in gut microbiota diversity commensurate with that of the healthy elderly population. Metformin has been linked with improved glucose uptake and the alteration of gut microbiota diversity [10, 11]. Elderly study participants who have diabetes practice similar dietary habits with the healthy elderly population, besides abstinence from animal protein, tuber and cassava products. Modification of the diet of elderly with T2D is likely to have led to an improvement in health. Previously, it has been reported that a diet low in proteins and carbohydrates improve glucose tolerance and overall health of individuals with T2D [5]. It is likely that reason for lack of observed difference in the alpha diversity of the gut microbiota in the studied cohort is that the modification of the diet is one of the factors contributing to the improved alpha diversity of individuals who have T2D to be commensurate with

that of healthy individuals. This is coupled with the fact that other factors such as age, ethnicity, lifestyle, and geographical location of the cohort are the same. The observed significant difference in gender and level of education between the study groups necessitated the consideration of these factors during the analysis of alpha and beta diversity. Nevertheless, they are not associated with the composition of the gut microbiota.

On the other hand, Wang studied healthy and T2D individuals within two minority ethnic groups in China and found that in one ethnic group (the Kazaks), the average number of observed OTUs (each OTU represents a microbial species) was significantly greater in the healthy population than in the T2D population. However, in the other ethnic group (the Uyghurs), there was no significant difference in the average number of observed OTUs in the healthy population and the T2D population [19], thus signifying that microbial community composition varies with ethnic group. Furthermore, within the Kazaks, significant microbial richness in the healthy group (compared to the diabetic group) was only observed at the family level, and not at the genus or species level. Based on these findings, it seems to be that alpha diversity of the gut microbiota at the genus and species level is less related to T2D than alterations in the stability of the gut microbiota [3]. It is worthy to note that in spite of the differences in the ages of study participants who participated in Wu's and Larsen's study (35–65 years, and 31–73 years, respectively), and the study participants in this study (60 + years), alpha diversity reports were similar. Nevertheless, to the best of our knowledge, this is the first study to report similar trends in an African elderly population.

Based on PERMANOVA and ANOSIM tests for associations between study groups and the composition of the gut microbiota (using Jaccard and Unweighted unifrac distances), the taxonomic composition of the gut microbiota in elderly individuals who have diabetes differs significantly from healthy elderly individuals, and are associated with the diabetic status of the study groups. This observation showed that although the gut microbiota alpha diversity within both study groups is similar, there is a clear distinction between a healthy microbiota and a gut microbiota associated with metabolic diseases such as T2D. As the characteristics of the study population are quite similar (besides the diabetic state), it is more than likely that the diabetic status plays a major role in the composition of the gut microbiota of elderly individuals within the two study groups. The findings are in agreement with previous reports on the compositional variation of the gut microbiota in T2D patients compared with healthy individuals [1, 3, 13, 14, 24]. For example, Larsen's study reported significant difference in the taxonomic beta diversity of the gut microbiota of healthy individuals and individuals with diabetes at the phylum and class taxonomic level [14]. The findings suggest that the diabetic state is associated with alterations in the gut microbiota composition in individuals who have diabetes, and that these alterations play a greater role in the evolution of diabetes than gut microbiota taxonomic alpha diversity. To the best of our knowledge, this is the first study to report gut microbial community differences in a healthy African elderly population and elderly subjects with diabetes.

Although no significant difference in the overall predicted functionality of the gut microbiota between the study groups was observed in this study, predicted functional pathways/features were differentially abundant in healthy urban elderly than in diabetic elderly. By prediction of functional capacity of the gut microbiota with 16S rRNA gene sequence data, we observed the enrichment of pathways involved in the biosynthesis of colonic acid building blocks, GDP-mannose, and GDP-Mannose-derived O antigen building blocks in the healthy urban elderly respondents. Colanic acid is an extracellular polysaccharide usually found in members of the *Enterobacteriaceae* [34]. O antigen is one of the components of lipopolysaccharide [35], the primary component of the outer membrane in bacteria which functions in shielding the bacterium against chemicals and modulating the immune system. It is likely that the biosynthesis of both compound's building blocks is essential for the maintenance of the cell integrity of individual members of the gut microbiota community, which could have enhanced microbial community homeostasis in the gut of the healthy elderly

volunteers. Furthermore, pathways involved in urea cycle and aerobic respiration (cytochrome c) were differentially abundant in healthy urban elderly respondents. Within the urea cycle, metabolic wastes resulting from amino acid catabolism are converted to ammonia, and then to urea. Some microorganisms within the *Clostridiaceae* family (including *Clostridium*) partake in the fermentation of amino acids and hydrolysis of the final byproduct of amino acid fermentation (urea) into ammonia [36], which further supports the enrichment of members of *Clostridiaceae* in the gut of healthy elderly. Excess ammonia is excreted in the faeces, while the rest serves as the building block of amino acid biosynthesis. These pathways seem to help mammals to eliminate urea as well as foster healthiness. On the other hand, S-adenosyl-L-methionine and L-lysine biosynthesis were the only predicted functional pathways enriched in elderly individuals with diabetes. The biosynthesis of L-lysine has been shown to be down-regulated in association with insulin resistance [37], suggesting that it is negatively associated with T2D. However, lysine is one of the essential amino acids associated with increased risk of diabetes [38, 39]. On the other hand, S-adenosyl L-methionine biosynthesis is negatively associated with the occurrence of diseases, because S-adenosyl L-methionine enzymes are essential components in the body as they catalyze numerous and varied chemical reactions [40]. However, in individuals who suffer from one of the co-morbidities of diabetes, nephropathy in particular, S-adenosyl L-methionine seems to be one of the risk factors for kidney failure [41]. It is likely that both amino acids are beneficial in the early stages of diabetes, but increase the risk of diabetes-associated co-morbidities in advanced stages of diabetes. Together with microbial biomarkers associated with diabetes, these amino acid biomarkers could inform the best course of action to take in the treatment of diabetes before the onset of diabetes-associated co-morbidities.

Our study has certain limitations. First of all, due to multifactorial influences on the gut microbiota in health and disease, it is imperative that the associations between various factors and the gut microbiota are investigated. We investigated the influence of lifestyle (including diet), age, gender, level of education, and occupation on the composition and predicted function of the gut microbiota. However, we did not look critically into the impact of other important factors such as body mass index (BMI) and the use of drugs such as metformin (the drug of choice in diabetes treatment) on the gut microbiota in the study participants. These factors might have influenced the gut microbiota of the study groups. Also, we acknowledge that metabolic predictions based on PICRUSt analysis of 16S rRNA gene sequence data may not necessarily capture the actual metabolic capacity of the gut microbiota. As such, cautious interpretation of information from metagenomic prediction is essential.

In conclusion, our study provided new and relevant information on the variation of the gut microbiota in association with the diabetic state in elderly populations. Some of the relevant information include evidence of gut microbiota differences (beta diversity) in healthy urban elderly compared with elderly individuals living with diabetes. Furthermore, taxonomic microbial biomarkers such as the enrichment of *Ruminococcus* and members of the phylum Actinobacteria (family *Bifidobacteriaceae*) in the elderly living with diabetes has not been reported previously. The enrichment of members of *Clostridiaceae* (*Clostridium*) and *Peptostreptococcaceae* in the healthy urban elderly corroborates findings from other studies which show the importance of these microbial markers to health. Also, we carried out the first study of its kind in an African continent as we focused our study on a relatively homogenous population of the elderly, an essential group of the human race to which beams of research light should fall on, if we intend to improve continental and global life expectancy. Information derived from this study and subsequent large-scale studies are needed to guide actionable policies aimed at restoring the gut microbiota of elderly individuals who have diabetes back to the healthy state, in order to prevent the elderly from suffering from increasing burden of diseases.

#### 4. Methodology

**Ethics:** The study was approved by the University of Ibadan/University College Hospital (UI/UCH) ethical review committee on the 9<sup>th</sup> of September, 2016 with reference number UI/EC/16/0211. Prior to sample collection, written informed consent and verbal informed consent were obtained from educated and non-educated volunteers, respectively.

**Study Design:** This is a cross-sectional study of healthy elderly individuals and elderly individuals who have diabetes (60 years old and above). This study was conducted over a 3-month period from the 10<sup>th</sup> of September, 2016 until the 20<sup>th</sup> of December, 2016.

**Recruitment of Participants:** Participants included twenty elderly individuals who were previously diagnosed patients with T2D (61–81 years) managed on an out-patient basis at the at the Chief Anenih Geriatric Centre, located within the University College Hospital premises, Ibadan, Nigeria. Ibadan is the largest city in West Africa, and is home to one of the major ethnic groups in Nigeria, the Yoruba. Routinely, elderly individuals who have diabetes attend clinic once in 3 months for medical check-up. Also, twenty-two consenting healthy elderly volunteers (60–80 years) living in the same city (Ibadan) were also recruited for this study. Healthy individuals who had received antibiotics for at least one month before sampling were excluded from participating in the study. Written informed consent was obtained from the subjects enrolled.

**Data Collection:** Dietary and medical information on the participants of the study were obtained with the use of an interviewer-administered structured questionnaire (a combination of questionnaire on dietary habits, lifestyle, level of education, ethnicity, and medical history (drugs used at least within the months prior to the commencement of the study)). Differences in age, sex, educational status, ethnicity, and occupation were tested using the Mann-Whitney U test (<https://www.socscistatistics.com/tests/mannwhitney/default2.aspx>) and the Chi Square test (<https://www.socscistatistics.com/tests/chisquare2/default2.aspx>). Based on the statistical significance of sex/gender ( $p = 0.03$ ) and occupation ( $p = 0.01$ ), these two variables were critically investigated with regard to alpha and beta diversity for human groups studied. None of these calculations resulted in a statistically significant result, see [Supplementary Table S1](#) and [Table S1](#).

**Collection of Samples and Storage:** The subjects were given sterile wide 50 ml bottles and instructions for providing faecal samples. After collection, the samples were handled and stored following previously described methods [42, 43]. Briefly, stool samples were aseptically submerged into 30 ml of 97% ethanol within a sterile container for 24–36 h. Ethanol (95–97%) has been shown to preserve stool samples and inherent microorganisms effectively for metagenomic and metabolomic studies, in instances where immediate fresh stool sample processing is impossible [44, 45]. The ethanol was carefully decanted and the solid material was transferred into 3mm-silica-gel-bead-containing tubes, while a sterile cotton wool was placed just above the beads (for desiccation). All samples were transported by express to the Medical University of Graz, Austria. They were then stored at  $-80^{\circ}\text{C}$  for further analysis.

**Extraction of DNA from Samples, Library Preparation of 16S rRNA Gene, and Sequencing with Illumina:** DNA extraction of the total microbial community genome was carried out following the methods of Klymiuk [46], with the use of MagNA Pure LC DNA III Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's protocol (Manual v13, November 2012). The primer set F515 (5'-GTGCCAGCMGCCGCGTAA-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3') (MWG, Ebersberg, Germany) was used for 16S rRNA gene amplification and library preparation steps according to the method of Klymiuk [46]. PCR reaction for each sample was done in triplicates. Sequencing was achieved using the Illumina MiSeq (Illumina, Eindhoven, Netherlands).

**Analysis of Processed Sequence Data:** Sequenced data in fastq format were imported into the QIIME2 pipeline (version 2019.7, <https://docs.qiime2.org/2019.7/>) [47], quality-controlled with the q2-dada2 plugin (<https://github.com/qiime2/q2-dada2>) [48], and explored for

downstream analysis with the FeatureTable artifact. A rooted phylogenetic tree needed for alpha diversity analysis was generated with the q2-phylogeny plugin (<https://github.com/qiime2/q2-phylogeny>). Reads from each sample were rarefied to a depth of 10000 in order to minimize the effect of sequencing depth on alpha and beta diversity measures. Alpha diversity and beta diversity analyses were achieved through the q2-diversity plugin (<https://github.com/qiime2/q2-diversity>) along with the R packages qiime2R, tidyverse, and ggpubr. Furthermore, analysis of similarity (ANOSIM), Adonis, and Permutational multivariate analysis of variance (PERMANOVA, based on beta diversity distance matrices and 999 permutations) were conducted within the QIIME2 pipeline through the q2-diversity plugin in order to analyze sample composition in the context of categorical metadata, and to disclose the factor(s) responsible for the variation in the composition of the gut microbiota in the study groups. The Calypso, an online user-friendly platform, was used to view the beta diversity analysis ordination plots [49]. Also, the emperor plugin enabled the interactive view of beta diversity ordination plots (<https://github.com/qiime2/q2-emperor>) [50].

Taxonomic and compositional analysis were achieved with the use of the plugins q2-feature-classifier (<https://github.com/qiime2/q2-feature-classifier>), a pre-trained Naïve Bayes classifier, and q2-taxa (<https://github.com/qiime2/q2-taxa>). The Greengenes 13.8 99% OTUs database (<http://greengenes.secondgenome.com/>) specific for the V4 region (515F/806R primers) of the 16S rRNA gene was used to train the classifier and to assign the taxonomy. Discriminant microbial features between the study groups were determined with the use of linear discriminant analysis (LDA) effect size (LEfSE) on the Galaxy platform (<https://galaxy.medunigraz.at>,  $p < 0.05$ , LDA score  $> 3.0$ ).

The prediction of functional capability from 16S rRNA sequence data was conducted by using the q2-picrust2 plugin (<https://github.com/picrust/picrust2/>). Following rarefaction (sampling depth: 739932), functional alpha and beta diversity metrics were calculated from the picrust2 output with the use of the q2-diversity plugin (<https://github.com/qiime2/q2-diversity>). Discriminant functional features of the microbial community between the study groups were determined with the use of linear discriminant analysis (LDA) effect size (LEfSE) on the Galaxy platform (<https://galaxy.medunigraz.at>,  $p < 0.05$ , LDA score  $> 2.0$ ).

#### Declarations

##### Author contribution statement

A.O. Afolayan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

L.A. Adebuseye: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

E.O. Cadmus: Contributed reagents, materials, analysis tools or data.

F.A. Ayeni: Conceived and designed the experiments; Wrote the paper.

##### Funding statement

This work was supported by an Austrian Agency for International Cooperation in Education and Research (OEAD-GmbH). postdoctoral fellowship awarded to Afolayan AO tenable at Medical University of Graz, Graz, Austria.

##### Competing interest statement

The authors declare no conflict of interest.

##### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2020.e03459>.



## Acknowledgements

We acknowledge the contributions of Christine Moissl-Eichinger, Christoph Hoegenauer, Gregor Gorkiewicz and Bettina Halwachs in sequencing data processing, laboratory procedures and project administration. We appreciate Ingeborg Klymiuk and Alexandra Benharkou for their help with the protocols and hands-on training. Support for this research was obtained from the Austrian Agency for International Cooperation in Education and Research (OEAD-GmbH) through a post-doctoral fellowship to A.O. Afolayan. For sequence data processing, the authors acknowledge the support of the ZMF Galaxy Team: Core Facility Computational Bioanalytics, Medical University of Graz.

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