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Oral microbiome characterization in patients with type 2 diabetes mellitus and diet as an influencing factor

Ana Cristina F. Almeida Santos

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Oral microbiome characterization in patients with type 2 Diabetes Mellitus and diet as an influencing factor

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Mestrado em Ciências do Consumo e Nutrição

Departamento de Geociências, Ambiente e Ordenamento do Território

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

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"Quero percorrer todas as páginas que hoje adivinho

Serem o meu destino. Não uma meta, mas um caminho,

Não um discurso, mas uma voz.

Descer um rio por capítulos e no fim dessas viagens

Ter encontrado o meu destino, não na foz,

Mas ao longo das margens."

- José Almeida

"Imagination will often carry us to worlds that

never were. But without it we go nowhere."

-Carl Sagan

Resumo

O microbioma oral é talvez um dos mais diversos e dinâmicos do organismo humano sendo possível identificar mais de 700 espécies de microrganismos. Estes microrganismos, em situações normais, vivem em equilíbrio com o hospedeiro, contudo, diversos fatores podem estar na origem de alterações ambientais e consequentemente resultar em disbiose. A disbiose pode estar na origem do aparecimento de doenças orais prevalentes como cáries e periodontite. A periodontite tem vindo a ser relacionada com a Diabetes de Mellitus tipo 2, sendo esta relação disfuncional nos dois sentidos, dado que estas duas doenças se influenciam mutuamente. São vários os estudos que demonstram que o microbioma oral está na base desta associação, para a qual a dieta pode ter um papel significativo enquanto modelador do microbioma oral. Contudo, os estudos que caracterizaram o microbioma oral em diabéticos tipo 2 são escassos e nenhum, até ao momento, usou a população portuguesa como objeto de estudo.

O objetivo principal deste estudo é caracterizar o microbioma oral de pacientes diabéticos tipo 2 de Portugal e, avaliar possíveis associações entre a dieta e o microbioma oral, por metagenómica. O microbioma oral de 22 pacientes diabéticos tipo 2 e de 25 indivíduos saudáveis foi analisado através da sequenciação massiva da região V3-V4 do gene 16S rRNA. Foi investigada a composição do microbioma oral assim como a diversidade intra e interindividual. As medidas de diversidade incluíram a diversidade alfa (índice de Shannon e abundância de “polimorfismos de sequencias” e a diversidade beta (dissimilaridade de Bray-Curtis). O microbioma oral entre o grupo controlo e os diabéticos foi comparado através do teste Permanova e da análise de componentes principais. Complementarmente foram exploradas as possíveis associações entre a constituição do microbioma oral e os hábitos alimentares.

No total, foram encontrados 233 taxa, 202 pertencentes ao grupo controlo e 183 ao grupo de diabéticos, dos quais apenas 65% eram partilhadas entre os dois grupos. O número médio de taxa por individuo foi 54, e apenas foi possível identificar até ao nível espécie 21 taxas dos 233. Não foram encontradas diferenças ao nível da diversidade alfa, beta e na composição da microbiota oral entre os dois grupos de indivíduos. Contudo, foi possível denotar algumas diferenças taxonómicas ao nível da classe, género e espécie. Ao nível da classe, as diferenças mais acentuadas foram a abundância das classes *Gammaproteobacteria,* que apareceu em maior quantidade no grupo controlo (11.2%) do que no grupo dos diabéticos (5.4%) e, *Betaproteobacteria* que foi mais abundante nos diabéticos (9.2%) quando comparado com o grupo controlo (4.2%). Adicionalmente, *Synergistia* foi a única classe significativamente diferente entre

os dois grupos, mesmo após ter sido aplicado o teste de correção de Bonferroni. Quanto ao nível do género, a abundância do táxon *Neisseria* foi maior no grupo de diabéticos do que no grupo controlo. A dieta demonstrou ser um fator influenciador no microbioma oral, onde foi possível inferir associações entre alguns componentes da dieta e a presença de alguns taxa.

Este estudo é o primeiro a revelar a composição taxonómica do microbioma oral de diabéticos tipo 2 portugueses, assim como de possíveis associações entre a dieta e o microbioma oral numa amostra da população portuguesa.

Palavras-chave: microbioma oral, metagenómica, diabetes de mellitus tipo 2, dieta, sequenciação do gene 16S rRNA

Abstract

The oral microbiota represents one of the most diverse and dynamic microbiotas of the human body where it is possible to identify more than 700 species of microorganisms. These microorganisms in normal situations live in harmony with the host; however, some factors can trigger ecological changes and consequently generate dysbiosis, which can lead to the onset of oral diseases, such as caries and periodontitis. Periodontitis has been related to type 2 diabetes mellitus, where both diseases engage in a two-way dysfunctional relationship. Several studies have demonstrated that oral microbiome is a driver in this association. Simultaneously, diet also has a significant role in modeling the oral microbiome. However, few studies have characterized the oral microbiome in type 2 diabetics, and none have been carried out on the Portuguese population.

The main objective of the present study is to characterize the oral microbiome of type 2 diabetes mellitus patients from Portugal and to evaluate possible associations between diet and the oral microbiome, using metagenomics. The oral microbiome of 22 participants with type 2 diabetes mellitus and 25 healthy subjects was examined using 16S rRNA gene amplicon sequencing of the hypervariable V3-V4 region. We investigated the oral microbiome composition and its diversity intra and inter individuals. The diversity measures included alpha diversity (Shannon index and Amplicon Sequence Variants abundance) and beta diversity (Bray-Curtis dissimilarity). We compared the oral microbiome of diabetics and control groups with a Permanova analysis and Principal Component Analysis. Also, we explored possible associations between the oral microbiome and dietary habits.

A total of 233 taxa were found, 202 present in the control group and 183 in the diabetes group, being 65% of the taxa shared by both groups. The mean number of taxa per individual was 54. Only 21 of the 233 taxa were taxonomically assigned as species. No differences were found between the control group and the diabetes patients in terms of alpha and beta diversity. Although no significant differences in the oral microbiome of control and diabetes patients were found, it was possible to perceive some minor taxonomic differences at the class, genera and species level. The main difference was that *Gammaproteobacteria* had a higher abundance in the control group (11.2%) than in the diabetes group (5.4%), whereas *Betaproteobacteria* was more abundant in the diabetes group (9.2%) when compared to the control group (4.2%). Additionally, *Synergistia* was the only class that was significantly different between groups, even after Bonferroni correction test. At the genus level, *Neisseria* abundance was greater in the diabetes group as well as the species level. Diet exhibited to be an influencing factor on

the oral microbiome since it was possible to infer some associations between dietary components and the presence of some taxa.

This study is the first to provide the taxonomic composition of Portuguese type 2 diabetes patients, and also possible associations between diet and the oral microbiome for a sample of the Portuguese population.

Keywords: oral microbiome, type 2 diabetes mellitus, metagenomics, 16S rRNA gene sequencing, diet

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List of Abbreviations

ASV	Amplicon Sequence Variant
bp	Base pairs
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DRIs	Dietary Reference Intakes
FFQ	Food Frequency Questionnaire
HMP	Human Microbiome Project
LPS	Lipopolysaccharide
µl	Microliter
mM	Millimolar
min	Minutes
nM	Nanomolar
NGS	Next-generation sequencing
OTU	Operation taxonomic unit
PCA	Principal component analyses
PCR	Polymerase Chain Reaction
PREVADIAB	Prevalence of the diabetes in Portugal
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic Acid
SFA	Saturated Fatty Acid
SPSS	Statistical Package for the Social Sciences
s	Seconds
T2DM	Type 2 Diabetes Mellitus
WHO	World Health Organization

1. Introduction

The microorganisms that reside in the human body are mainly Bacteria, Archaea, Fungi and Viruses, which outnumber human cells by about ten to one, that is, our own body has more microorganisms than human cells (Group et al., 2009). The ecological community of microorganisms that resides in an established environment is referred to as the microbiota, while the term microbiome is attributed to the microbiota's collective genomes (Turnbaugh et al., 2007). It is worth noting that human microbiota is composed predominantly by bacteria populations (Petrosino et al., 2009) and so, the majority of microbiome studies are focused on this group.

The human body provides various microhabitats, such as oral cavity, skin and gut that contribute to the overall microbiota (Badger et al., 2011; Sonnenburg & Fischbach, 2011). Each one provides unique environmental conditions such as temperature, salinity, pH and access to nutrients and oxygen, therefore each habitat has its exclusive microbiota (Struzycka, 2014). Besides, the microbiome is more similar between different individuals when comparing the same body location than when different locations on the same individual are compared (Sonnenburg & Fischbach, 2011).

Normally, the microorganisms are engaged in a mutualistic relationship with their host. Nonetheless, the composition of these resident communities can change, leading to an ecosystem imbalance or, in other words, culminating in dysbiosis (Lloyd-Price et al., 2016). Dysbiosis has been related to several diseases such as diabetes (types 1 and 2), allergies (Trompette et al., 2014), asthma (Abrahamsson et al., 2014; Ver Heul et al., 2018), inflammatory bowel disease (Frank et al., 2007) and cancer (Garrett, 2015). Thus, being able to distinguish between healthy or unhealthy microbiota becomes a fundamental tool to fully understand human health and disease (Dewhirst et al., 2010).

1.1. Brief history of microbiotas' studies

The interest in the microorganisms can be traced back to Leeuwenhoek who first reported the existence of microbiotas (Hamarneh, 1960). After Leeuwenhoek, and for almost 300 years, microbiota studies were mostly based on the use of microscopy and culture techniques. The need to develop the ultimate medium to isolate and cultivate microorganisms in a laboratory was a *sine qua non* condition to study a microorganism. During the last century, an uncountable number of works were made to describe, a yet, uncountable number of microorganisms. But not all microorganisms can grow in a laboratory, and therefore these culture-dependent methods become a major caveat on

the detection and characterization of the living organism of the microworld (Escobar-Zepeda et al., 2015; Morgan & Huttenhower, 2012).

In a need to surpass these limitations, the development of the molecular biology in the last 30 years, propelled the emergency of nonculture methods and greatly improved the microorganism accountability. One of those techniques, the polymerase chain reaction (PCR), which allows amplifying targeted genes, had an enormous impact. However, there was still a lack of detail associated to these methods such as the understanding of microbiome correlations with the environment (Escobar-Zepeda et al., 2015) and this, together with the impossibility of PCR amplification from deeply divergent and unknown organisms, kept the pressure for the development of new techniques.

Finally, several technological advances during the first decade of this millennium led to the appearance of equipment able to combine cutting-edge technology with new DNA sequencing methods, which led to the creation of protocols that can massively sequence the DNA molecules present in a given biological sample. These new techniques were then baptized as next-generation sequencing (NGS), and become responsible for the inauguration of the genomics era (Escobar-Zepeda et al., 2015; Morgan & Huttenhower, 2012).

The base of the NGS technologies is the non-specificity of the sequencing of all the DNA that exists in a given template. As all living beings are also the habitat of thousands of other microorganism species, when a biological sample from a given specimen (e.g., human) is taken, it contains a sample of those microbiological organisms that populate that specimen plus its own DNA. Consequently, when that sample is sequenced by NGS technology, besides the sequencing of the targeted specimen genome, the genomes of the microorganisms presented in that sample, will be also sequenced (Herzyk, 2014). The collection of the genomes of all the microorganisms living in a host is referred as the third genome, called the metagenome (Escobar-Zepeda et al., 2015; Herzyk, 2014).

Therefore, metagenomics analyses consist of the recovery of all the genomes a given microbial community sampled from its natural environment. Metagenomics provides insights not only about which microorganisms are present in a given environment but also about microbiome diversity. The two main approaches for determining the microbiome diversity are the shotgun sequencing and the amplicon sequencing of the 16S ribosomal RNA (16S rRNA) (Escobar-Zepeda et al., 2015). Shotgun sequencing consists of breaking randomly all the DNA sequences contained in a sample, forming small fragments of DNA that will be reassembled based on overlaps (Parla et al., 2011). On the other hand, the 16S rRNA is composed of conserved regions that are used to design PCR primers for amplification of hypervariable regions that are unique to each

bacterial species, allowing bacterial specific identification. There are nine hypervariable regions (V1-V9), which display different degrees of diversity. It is not possible to sequence all the 16S rRNA gene in one PCR reaction, due to its length, therefore the selection of the most efficient hypervariable regions becomes crucial. There is no consensus on which regions to use, however, several studies have demonstrated that some regions are more sensitive than others (Fuks et al., 2018; Yang et al., 2016). Shotgun sequencing is a more expensive and complex method than 16S rRNA, however, it provides a better taxonomic resolution (Farina et al., 2019). Due to the relative easiness of amplification and sequencing of the 16S rRNA gene, it has been widely used in microbial studies for taxonomic assignment (Belda-Ferre et al., 2012; Mitreva, 2017). For both the shotgun and the 16S rRNA gene approaches, the sequencing is carried out using next-generation sequencing technologies, which enable to sequence millions of DNA fragments simultaneously and independently. After sequencing, bioinformatics analyses are used to process the DNA fragments (reads) and map them to reference genomes (Behjati & Tarpey, 2013).

NGS advances have made feasible the performance of large-scale studies such as the Human Microbiome Project (HMP). In 2008, the HMP was established with the purpose to characterize the microbial communities found at different sites on the human body, to enable a more comprehensive understanding of the human microbiome (Kilian et al., 2016; Zarco et al., 2012).

Despite NGS technologies being a powerful tool in a variety of fields such as human genetics, disease gene identification, microbiology and others (Behjati & Tarpey, 2013), they also have some limitations and biases. These technologies require programming and scripting knowledge for running and installing the metagenomics software and also for data analyses and interpretation. In addition to bioinformatics complexity, it is also required a high computational capacity and storage due to the enormous dataset generated (Behjati & Tarpey, 2013; Pereira et al., 2017). Furthermore, NGS technologies still need to be improved to avoid bias in sequencing some species over others (Ross et al., 2012), as well as enhancing and completing some microorganisms' databases, particularly related to virus', which is still very incomplete (Strazzulli et al., 2017).

1.2. Oral microbiome

The oral cavity is the main gateway for microorganisms' entrance into the human body. They enter through the nose and mouth thought breathing. Also, they enter through the mouth within the food we ingest and, after food is chewed and mixed with saliva,

continues through the gastrointestinal tract (Silva, 2016). Furthermore, the oral cavity is a heterogeneous environment (i.e., ecosystem) due to the variety of distinct habitats such as teeth, gingival sulcus, tongue, cheeks, hard and soft palates and tonsils, where each one provides specific conditions and nutrients for its microbiota. Hence, some bacteria are site-specific such as *Rothia,* which inhabits the tongue and tooth surfaces, *Streptococcus salivarius* that mostly colonizes the tongue, and *Simonsiella* colonizes the hard palate. However, some bacteria can be detected in all oral sites, such as species of *Streptococcus*, *Neisseria*, and *Prevotella* (Aas et al., 2005; Kazor et al., 2003; Mager et al., 2003).

Considering the continuous entrance of microorganisms and the high diversity of habitats provided by the oral cavity, the oral microbiota represents one of the most diverse and dynamic of the human body, in which it has been possible to identify more than 700 species of bacteria (Long et al., 2017).

Oral microbiota colonization begins immediately after birth and its composition develops during the first years of an infant's life (Drell et al., 2017). Commonly, *Streptococcus* and *Actinomyces* genera are the first colonizers, followed by *Veillonellae* and *Fusobacteria* genera (Koenig et al., 2011; Kononen, 2000). Nonetheless, maternal factors influence infants' oral microbiota development, such as the type of delivery and the feeding regime (Zaura et al., 2014). Vaginally born infants harbor oral bacterial communities similar to their mothers' vaginal microbiota and infants born via cesarean section (C-section) exhibit an oral microbiome that resembles the microbiota from their mothers' skin (Dominguez-Bello et al., 2010). Additionally, those born from C-section acquire *Streptococcus mutans* nearly a year earlier than vaginally delivery infants as well as show a lower taxonomic diversity at 3 months of age, probably due to less exposure to the maternal microbiota at birth (Li et al., 2005; Lif Holgerson et al., 2011). Although little information exists regarding the influence of breastfeeding on the composition of the oral microbiota, some studies have demonstrated that breast-fed infants carried oral lactobacilli with antimicrobial properties, which inhibit growth and adhesion of cariogenic bacteria like *S. mutans*. In contrast, these shreds of evidence were not found in formula-fed infants (Holgerson et al., 2013; Wernersson et al., 2006; Zaura et al., 2014).

Until adulthood, the oral microbiome starts to mature and becomes significantly more diverse (Lif Holgerson et al., 2015). Additionally, alongside with this gradually transformation, potentially cariogenic bacteria seem to emerge (Crielaard et al., 2011; Yang et al., 2012).

Throughout adulthood, the oral microbiome remains stable under healthy conditions. Nevertheless, ageing process can affect the microbiota's composition, leading to a diversity loss, causing an increase of pathogenic bacteria and changing the dominant species. Various studies have reported that the prevalence of *Aggregatibacter actinomycetemcomitans* decreases with age while *Porphyromonas gingivalis* increases. Factors as diet, lifestyle, medication, mainly antibiotics (elderly people have an increased tendency to inflammation), denture wear, saliva flow, several diseases and a poor immune system tend to affect the microbiota. Some authors reported that denture wear increases the incidence of acidophilic bacteria like lactobacilli and yeasts in saliva (Ticinesi et al., 2018).

As reported by previous studies, bacterial phyla Firmicutes, Actinobacteria, Fusobacteria, Proteobacteria, and Bacteroidetes dominate the oral microbiota, accounting for 80-95 % of the total species (Yang et al., 2012). These microorganisms normally harmoniously co-exist with their host due to coevolution, however, behavioral factors such as poor oral hygiene and diet, a compromised immune system, genetics, and medication can lead to a dysbiotic oral ecosystem (Nath & Raveendran, 2013). This microbial imbalance is normally associated with an overgrowth of pathogenic microorganisms which can lead to more susceptibility to oral illness such as dental caries and periodontal disease (Nakajima et al., 2015; Woelber et al., 2016). Periodontitis, the severest form of periodontal disease, is the chronic inflammation of periodontal tissues, stimulated by the continuing presence of the biofilm (dental plaque) (Preshaw et al., 2012). The presence of *Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola* in the oral cavity seems to be an indicator of periodontitis (Griffen et al., 2012; Hong et al., 2015; Kirst et al., 2015). Furthermore, this disease has been associated with Diabetes Mellitus (DM).

1.3. Diabetes Mellitus

Diabetes Mellitus is a metabolic disease characterized by chronic hyperglycemia, caused by defects in insulin secretion, insulin action, or both. Consequently, this increase in glucose concentration in the bloodstream is responsible for many of the body systems' damage (World Health Organization, 2006) .

There are two main types of diabetes mellitus, type 1 and type 2. In type one, there is autoimmune destruction of pancreatic islet ß-cells and therefore there is no insulin production. It is typically present in children and adolescents. In type 2 diabetes mellitus (T2DM), the secretion of insulin is produced in a deficient manner, which can contribute

to the development of resistance to its action (Kuo et al., 2008; Mealey et al., 2006). The more prominent risk factors are diet, genetics, obesity and advancing age (Wu et al., 2014). T2DM is becoming more common among children and adolescents due to increasing childhood obesity rates. According to the World Health Organization (WHO), the prevalence of diabetes mellitus has been increasing exponentially with type 2 diabetes being responsible for more than 90% of these cases (Salunkhe et al., 2018), hence, this disease has become a significant global health care problem (Issa, 2017).

In conformity with the Annual Report of the National Diabetes Observatory, the Portuguese population, in 2005, exhibited an estimated prevalence of diabetes of 13.3% in individuals between the ages of 20 and 79 years (7.7 million individuals). That is, more than one million Portuguese individuals in this age group suffer from diabetes. Considering population aging repercussions on the prevalence of diabetes, it is estimated that between 2009 and 2015 the prevalence in the national territory increased 13.5%, and about 44% of the affected Portuguese population had not yet been diagnosed (Portuguese Society of Diabetology, 2016).

On the other hand, according to the National Health Examination Survey (INSEF 2015), the diabetes prevalence in the Portuguese population aged between 25 and 74, is 9.8%, which is higher than the European average (9.1%) (INSEF, 2015).

Concerning sex, men have a higher prevalence of diabetes (15.9%) than women (10.9%), being this difference statistically significant. Also, there is a positive correlation between the prevalence of diabetes and aging. More than a quarter of the Portuguese population between the ages of 60 and 79 years has diabetes (Portuguese Society of Diabetology, 2016). Similarly, there is also a relationship between BMI and diabetes and, according to the data collected under the national program “Prevalência da Diabetes em Portugal (PREVADIAB)”, 90% of the population with diabetes is overweight or obese, noting that an obese person displays a risk four times higher of developing diabetes than a person with normal weight (Gardete-Correia et al., 2010).

Diabetes prevalence is different among Portugal regions. Data from 2015 indicate that the highest value prevails in the Alentejo region (11.3%) and the lowest in the Algarve region (7.7%). It is also worth noting that Alentejo has also the highest prevalence of obesity in the continent and Algarve has the lowest. Additionally, the North region presented 9.5% of diabetes prevalence (Programa Nacional para a Diabetes, 2017).

The incidence rate of diabetes (the number of new cases per year) has increased largely in the last decade. In 2015, between 591 and 699 new cases per 100,000 inhabitants

were estimated. However, on a positive note, the mortality rate for diabetes has been decreasing, with the year 2015 having the lowest standardized mortality rate. Diabetes has a significant contribution to the causes of death and is currently responsible for more than 4% of women's deaths and more than 3% of deaths in men, that is, per year approximately 2,200-2,500 women and approximately 1,600-1,900 men die because of diabetes (Portuguese Society of Diabetology, 2016).

People who suffer from diabetes have an increased risk of developing several health problems for having consistently high blood glucose levels. The main chronic injuries are separated into microvascular complications (neuropathy, retinopathy, nephropathy) and macrovascular complications (cardiovascular disease and stroke). To delay or prevent diabetes complications, it is crucial to maintain the blood glucose levels, blood pressure, and cholesterol close to normal (Tschiedel, 2014). Hence, individuals with diabetes should be consistently monitored.

In the last decades, there have been several studies reporting that people with diabetes are also susceptible to oral problems due to an increased risk of inflammation of the gums. It was due to a study regarding the Pima Native American population (population with the highest incidence of diabetes type 2) (Mealey et al., 2006), in the 1990s, that T2DM became apparent as a risk factor for periodontitis. This study determined the incidence and prevalence of periodontal disease in 2,273 Pima individuals with more than 15 years old and verified that the prevalence of periodontitis was 60% in diabetic individuals and 36% in those without diabetes. The incidence was determined in subjects with slight or no sign of periodontitis at the beginning of the study, which was followed over 2.5 years. Diabetic patients displayed the triple of periodontitis incidence when compared to non-diabetic individuals (Nelson et al., 1990)

Despite the strong pieces of evidence that T2DM is a risk factor for periodontitis development, the causes are still inconclusive. However, it seems that poor glycemic control is an important factor in risk determination (Taylor et al., 1996).This hypothesis was first evidenced by an investigation of residents of the Gila River Indian Community. These individuals were aged 18 to 67, suffer from T2DM and were followed over two years. These authors found that the presence of severe periodontitis at the beginning of the study was related to poor glycemic control ((glycosylated hemoglobin (HbA1c) >9%), which in turn compromises the T2DM control (Taylor et al., 1996).

There could be a relationship between poor glycemic control and the oral microbiome since certain bacteria compromise its control. *Porphyromonas gingivalis,* one of the main strains of periodontal disease, produces a lipopolysaccharide (LPS) that is toxic to

certain proteins (cytokines) responsible for regulating insulin under normal conditions (Kuo et al., 2008; Makiura et al., 2008). However, other strains could be related to T2DM. Long et al. (2017) compared the oral microbiome profiles from 98 subjects with T2DM, 99 non-diabetic obese individuals and 97 non-diabetics with normal weight through 16S rRNA sequencing. They found that a higher abundance of most taxa in the phylum *Actinobacteria* were associated with a lower diabetes risk, where *Actinomycetaceae, Bifidobacteriaceae, Coriobacteriaceae, Corynebacteriaceae,* and *Micrococcaceae* families were less abundant among diabetic subjects compared to normal weight controls. Nevertheless, concerning the family *Actinomycetaceae*, Yacoubi (2013) verified that regarding severe periodontitis, *Actinomyces israelii* was exclusively identified in diabetic subjects and *Actinomyces naeslundii* was only detected in the control group. Additionally, another study found a decrease in the biological and phylogenetic oral microbiome diversity in diabetics in comparison to non-diabetic patients (Saeb et al., 2019). According to these authors, this evidence was related to an increase in the pathogenic content in the diabetic's oral microbiome. Although all these studies agree that differences between diabetic and non-diabetic oral microbiome exist, more studies are required to enlarge the current understanding of the association between the oral microbiome and this pathology. Additionally, apart from the fact that a population presents a particular genetic background, each population also has particular dietary and hygiene habits as well as diseases incidence and prevalence. This implies that the data cannot be extrapolated between populations. Therefore, studying different populations becomes extremely important.

1.4. Diet

Diet is one of the factors that affect the harmony of the oral microbiome since it influences the susceptibility to certain diseases, such as dental caries and periodontitis (Kilian et al., 2016; Warinner et al., 2015). As most of the human societies become more and more sedentary and urbanized, their contemporary diets have recently extremely diverged from the past and, for this reason, historical/ancient microbiome analysis could help to better understand the evolutionary patterns and changes across the times. This would ultimately help to enlarge the current understanding of the relationship between oral disease and oral microbiomes (Warinner et al., 2015).

The first significantly shift in the human diet occurred in the Neolithic period, characterized by agriculture emergence and, consequently, a substantial increase of cereals in the diet. Carbohydrates consumption is related to dental caries and periodontitis, diseases that were uncommon in pre-Neolithic populations (Dagli et al.,

2016; Kaidonis & Townsend, 2016). Dental studies on archaeological material dated from the Neolithic period have revealed a higher level of dental caries and periodontitis (Adler et al., 2013; Dagli et al., 2016; Warinner, 2016). One study that analyzed ancient European dental calculus samples found that the modern oral microbiome is less diverse and composed of more opportunistic cariogenic bacteria like *Streptococcus mutans.* Also, species such as *Porphyromonas gingivalis*, *Treponema,* and *Tannerella*, which are related to periodontitis manifestation, are more abundant in farming populations than in hunter-gatherer populations (Adler et al., 2013). Later, from the XVIIIth century onward, dental caries incidence increased as a result of the accessibility to refined flour and sugar (Kaidonis & Townsend, 2016). Presently, there is a growing body of evidence that demonstrate that a high-carbohydrate diet, mainly rich in sucrose, damages oral health. Individuals who frequently ingest carbohydrates, display a greater amount of acidophilic bacteria, particularly *Lactobacilli* and *Streptococcus mutans*, in the oral cavity (Hojo et al., 2009; Morhart & Fitzgerald, 1976; Wade, 2013). The acidic products produced by these types of bacteria are considered the main cause of dental caries (Gross et al., 2010; Wade, 2013)

Literature determining the correlation between dietary fat intake and the oral microbiome is still limited, although some studies have reported the influence of fat on the oral cavity homeostasis. It has been reported that the omega-6 / omega-3 ratio may be a periodontal disease predictor and frequent consumption of fatty foods has been associated with periodontitis in overweight individuals (Tomofuji et al., 2011). Also, a high-fiber and low-fat diet have been shown to improve periodontal disease markers alongside the bodyweight reduction (Kondo et al., 2014). According to Kato et al. (2017), all fatty acids showed identical tendencies regarding diversity indexes and bacteria groups, yet saturated fatty acids exhibited a more complex microbial community, in which several pathogenic bacteria were included.

Finally, a study comparing the oral microbiome from African populations showed that hunter-gatherers from the Batwa pygmy group, in Uganda, had greater bacterial richness and more profound differences in the abundance of 15 common genera of bacteria when compared to agricultural populations in Sierra Leone and the Democratic Republic of Congo (Nasidze et al., 2011). The authors of this study suggested that these differences could be related to Batwa's high-protein diet, where almost half of their diet consists of animal meat (Nasidze et al., 2011).

Regarding current diets, there are nutritional recommendations to guide the population on how to pursue a healthy diet. According to the Institute of Medicine of the National

Press, the Dietary Reference Intakes (DRIs) for daily macronutrient intake for adults, accounted for the total energy intake, are 40-65% from carbohydrates, 20-35% from total fat and 10-35% from protein. Protein intake can also be estimated by the multiplication of 0.8 grams of protein per kilogram of reference body weight. Furthermore, the intake of added sugar should be less than 25% of total energy and the intake of trans fatty acids and saturated fatty acids should be as low as possible while consuming a nutritionally balanced diet (Institute of Medicine Committee to Review Dietary Reference Intakes for Vitamin & Calcium, 2011).

Likewise, there are recommendations specific to certain diseases. In the case of individuals with diabetes, to improve overall health, the nutrition goals are centered on promoting and supporting healthy eating patterns, highlighting foods with high nutrient-density and its proper portions. Alongside these practices, body weight, glycemic, blood pressure and lipid profile goals can be attained. Lastly, nutrition therapy also has the goal of delaying or preventing diabetes complications (American Diabetes Association, 2019). Nevertheless, there is no dietary guideline that works universally for all diabetes patients, therefore, it is necessary to conduct an individualized nutritional therapy that can fulfill individual health goals and needs (Evert et al., 2019; Forouhi et al., 2018).

Even though there are no universal guidelines, there is consensus in some areas. Since type 2 diabetes is commonly related to overweight and obesity, one of the main objectives is weight loss and healthy weight maintenance. Weight loss improves glycemia, blood pressure and lipid levels; therefore, it could be a way to delay diabetes complications. According to the literature, weight loss should be achieved alongside with energy intake reduction and physical activity (Forouhi et al., 2018).

Concerning macronutrients, carbohydrates quantity and insulin levels seem to be the most imperative factors that influence the glycemic response, and it is advised to eat vegetables, fruits, whole grains and legumes over carbohydrates sources which contain added fats, sugars and sodium. Additionally, fructose that naturally occurs in fruit can result in better glycemic control than isocaloric intake or sucrose or starch (Forouhi et al., 2018).

Regarding total fat, the evidence is inconclusive whereas fat quality seems to be more important than quantity for a healthy diet. In any case, concerning fat quality the recommendation is that fat intake should be from monounsaturated and polyunsaturated fat, avoiding at all cost *trans*-fat and saturated fat. On the other hand, there is not an ideal amount of protein intake for type 2 diabetes patients, however, protein intake seems

to increase insulin response so, carbohydrates sources rich in protein should be avoided when treating or preventing hypoglycemia (Evert et al., 2014; Evert et al., 2019).

Since the oral microbiome is an important factor for human health maintenance, more studies need to be done to better understand this relationship and to promote future possibilities of treatment and diagnosis. Also, the promotion of healthy eating habits becomes crucial since diet affects oral cavity homeostasis.

2. Objectives

2.1. Main objective

Few studies on the relationship between type 2 Diabetes Mellitus with the oral microbiome are available and none has included the Portuguese population. Therefore, the main objective of the present study is to characterize the oral microbiome of type 2 diabetes mellitus patients in Portugal. Since oral microbiome can be influenced not only by diabetes but also diet, dietary habits were also taken into account, to roll out possible differences not related to diabetes type 2. To do so, we will compare the oral microbiome of T2DM patients to a control group and, evaluate possible associations between diet and oral microbiome as well.

With this study, we aim to conduct an exploratory and analytical study on the characterization of the oral microbiomes in individuals having type 2 diabetes using metagenomics.

2.2. Specific objectives

To fulfill the main objective, the following specific objectives were proposed:

- Characterize the oral microbiome by identifying the species of microorganism in each individual, as well as their abundance.
- Analyze microbiome diversity measures at the individual (alpha diversity), and the inter-individual (beta diversity) levels.
- Analyze the oral microbiome profiles and diversity patterns in both the diabetic and control groups and investigate if diabetes patients host specific taxa that are different from the control group.
- Evaluate dietary effects on the oral microbiome profiles.

3. Methods

3.1. Subject selection

The population sample consisted of twenty-five patients with Diabetes Mellitus type 2 (age range 51 to 75; average age 63) and twenty-five healthy individuals (age range 42 to 74; average age 60), recruited in Centro Hospitalar de Vila Nova de Gaia/Espinho. Regarding the sex ratio, both groups were composed of 17 males and 8 females.

Volunteers were ineligible if they presented any cognitive deficits, less than one-third of the dentition or were under 18 years of age. Volunteers under the influence of antibiotics for less than two months were also ineligible. The study was approved by Centro Hospitalar de Vila Nova de Gaia/Espinho's Ethics Committee and it was conducted according to the Declaration of Helsinki. Participants were given an informed sheet with all the relevant information about this study (Supplementary document 1). A written informed consent was acquired from each participant (Supplementary document 2).

3.2. Saliva sampling and questionnaire administration

All participants were approached after a general medicine or diabetic foot appointment and were previously instructed not to brush their teeth after their last meal before the examination. Each volunteer was submitted to a questionnaire (Supplementary document 3), through face-to-face interviews, regarding their lifestyle, including information on smoking, alcohol and coffee consumption, oral hygiene habits and physical activity. Information about food restrictions, what type of diseases they had, types of medications they were taking and the period time in the case of antibiotics, was also collected. Additionally, a validated semiquantitative food frequency questionnaire (Lopes, 2000; Lopes et al., 2007) was administrated to estimate nutrient intake in the last year (Supplementary document 4). Nutrient content was calculated using the Food Processor Plus (ESHA Research, Salem, Oregon) program. Lastly, each participant was asked to accumulate saliva in the floor of their mouth and then spit 2 milliliters into sterile test tubes which contained 2 milliliters of lysis buffer [50 ml of 1M Tris pH 8.0, 100 ml of 0.5 M EDTA, 50 ml of 1M Sucrose, 100 ml of 10% SDS and 50 ml of 2M NaCl] ((Quinque et al., 2006)). Each tube was placed in the cold (± 4ºC) for later analysis.

3.3. DNA extraction

DNA was extracted from saliva based on Quinque et al. (2006). Fifty microliters of proteinase K and 300 µl of 10% SDS were added to the tubes containing the 4 mL of

saliva and lysis buffer mixture, which was left to digest overnight at 53 °C in a shaking incubator. Then, 800 µl of 5 M NaCl was added and the tubes were incubated for 10 min on ice. After this, the mixture was distributed equally into 2-ml centrifuge tubes and centrifuged for 10 min at 14,000 rpm in an Eppendorf 5804R centrifuge. The supernatant from each tube was transferred to a new 2-ml centrifuge tube and 800 µl of isopropanol was added to each tube. Then, the tubes were incubated 10 min at room temperature and centrifuged for 15 min at 14,000 rpm. The supernatants were discarded, and the pellets were washed with 700 µl of 70% ethanol solution. Finally, the pellets were dried and dissolved in 40 µl of distilled and deionized water.

The isolated DNA was analyzed quantitatively and qualitatively using a Nano Drop-2000 spectrophotometer (Thermo Fisher Scientific Inc., MA USA) and agarose gel electrophoresis, correspondingly. All samples were stored at 4°C until further analysis.

3.4. 16S rRNA amplification, library preparation and sequencing

To amplify the V3-V4 hypervariable regions, the 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTACHVGGGTATCTAATCC-3') universal primers were used (Iriboz et al., 2018).

A two-step Polymerase Chain Reaction (PCR) method was used to first amplify the target region and then to attach a barcode to each individual to pool all the samples before sequencing.

The amplicon PCR contained 5 µl of DNA template at a concentration of 10 ng/µl, 5 µl of Taq PCR Master Mix kit (Qiagen), 0.4 µl of each primer and 3.2 µl of distilled and deionized water, in a final reaction volume of 14 µl per sample. The PCR cycling conditions were 95°C for 15 min, succeeded by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 30 s. The final elongation was run at 60°C for 5 min, followed by a hold at 15 °C. To control the quality and size of the amplicon, 2 µl of each amplified fragment was mixed with bromophenol blue and tested in 2% agarose gel with GelRed™ (Biotium) Nucleic Acid stain and visualized through a digital gel imaging system.

Secondly, a PCR clean-up by using AMPure XP beads (Beckman Coulter) was performed with a ratio of 0.6 µl of magnetic beads to 1 µl of PCR product, allowing the removal of primer-dimer and reagents that were not incorporated in the reaction. Then, the purified DNA was resuspended in 10 mM Tris pH 8.5. We verified the amplified

fragments with a 2% agarose gel with GelRed™ (Biotium) Nucleic Acid stain, expecting only a band corresponding to the amplicon size of 460 bp.

For the index PCR, a dual indexing strategy was performed, using two indices (i5 and i7) with 7 bp each. The reaction contained 5 µl of 2x Kapa HiFi Hot Start, 0.5 µl of each index, 2 µl of ultrapure water and 2 µl of DNA, in a final volume of 10 µl per sample. PCR cycling conditions were run at 95°C for 3 min, succeeded by 10 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. The final elongation was run at 72°C for 5 min, followed by a hold at 4 °C. To confirm the index incorporation, the samples were tested in a 2% agarose gel, expecting an amplicon with 650 bp, approximately. A second PCR clean-up was performed followed by library quantification using a Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific). All samples were normalized to 9 nM and pooled with 5 µl of each sample.

Finally, the TapeStation 2200 (Agilent Technologies) was used for the precise sizing and library quantification of the pool, followed by a library validation through a quantitative PCR.
The pool was sent to the NovoGene laboratory to be sequenced in an Illumina MiSeq sequencer with a 2x250bp paired-end configuration.

3.5. Sequence processing and alignment

Novogene provided the raw sequence data converted into forward and reverse reads in the format of fastq files, which were imported to the software “Quantitative Insights into Microbial Ecology” (QIIME) version 2-2019.7 (Bolyen et al., 2019).

The paired-end sequences were submitted to filtering, denoising, dereplication, chimera identification and merged through DADA2 (Callahan et al., 2016), a quality control package in QIIME2, with the default parameters. After quality control, the output that resulted from DADA2 was a feature table with amplicon sequence variants (ASVs), which registers the quantity of each ASV in each sample. Most microbiome studies have used operational taxonomic units (OTUs) to delineate microbial taxa. However, new methods have been developed such as the ASVs. OTUs are sequences clusters that differ by less than a dissimilarity threshold, normally 3%, that is, those OTUs sequences represent a certain taxonomic unit with 97% similarity. On the other hand, ASVs method can distinguish sequence variants differing by only one nucleotide, without imposing the OTUs threshold, which is expected to increase taxonomic resolution (Callahan et al., 2017).

For taxonomic assignment, we used the Naïve Bayes classifier trained on the Greengenes v13_8 99% OTUs databased, which is the microbial genomes databased used in the alignment of the reads. After a taxonomic assignment, the ASVs annotated as mitochondria and chloroplast were removed.

3.6. Statistical analyses

A bar plot for each taxonomic level was built to depict the taxonomic abundance in each sample. Relative frequencies of each ASV were also calculated for each taxonomic level and differences between the control and diabetes groups were tested using the Mann-Whitney U test in SPSS v.25. Finally, a heatmap and a hierarchical clustering was constructed using the *pheatmap* package from R (Kolde, 2019), to evaluate samples clustering. Clustering was completed using the Wards method.

Since subjects with diabetes seem to have a higher predisposition to periodontitis, the relative frequency of the bacteria associated with periodontal disease between groups was also investigated and compared through the Mann-Whitney U test calculated with SPSS v.25.

Microbiome diversity was evaluated using alpha-diversity, which expresses the richness (number of distinguishable taxa) and evenness (taxa distribution) within a sample and, beta-diversity, which indicates the differences in the taxa structure between samples. Alpha-diversity metrics included the ASVs abundance and Shannon diversity index (Shannon, 1948), whereas beta-diversity metrics were calculated through Bray-Curtis dissimilarity (Bray & Curtis, 1957). For the diversity analyses, we performed rarefaction curves to examine which sampling depth to use, with QIIME2 program.

The statistical significance of the comparison of alpha and beta diversities between the control and diabetes groups was calculated using the nonparametric Kruskal-Wallis H test and PERMANOVA with 999 permutations respectively, in the QIIME2. The PERMANOVA test if samples within a group are more similar to each other (within-group distances) than to samples from another group (between-group distances).

To investigate possible clustering of the samples analyzed, we performed a principal coordinated analysis (PCA) through EMPeror (Vazquez-Baeza et al., 2013) using the Bray-Curtis matrix.

Regarding diet, a descriptive analysis from both diabetes patients and the control group (50 individuals) was performed using SPSS V.25 (Supplementary table 1-2). The dietary analysis was limited to daily energy and macronutrients intakes, such as proteins,

carbohydrates and fats. Concerning carbohydrates and fat, individual analysis of sugar, monounsaturated, polyunsaturated and saturated fats were also performed (Supplementary table 1-4).

We also considered Body Mass Index (BMI), which was calculated by the weight in kilograms divided by the square of the height in meters (kg/m^2). The weight was measured with a scale (SECA) with an error of ± 0.5 kg. The BMI was divided into six categories based on a report of the World Health Organization (2000) for a better understanding of data distribution (Supplementary table 5-6).

To investigate if there were differences in nutrients consumption, energy intake, and BMI, between the control group and diabetes patients, a Mann-Whitney U test was applied or a Student's T-test when the data followed a normal distribution. Both tests were performed with a 5% level of significance. All multiple tests p-values were adjusted through the Bonferroni correction test.

Finally, we used Spearman correlations to identify possible associations between diet and the obtained taxa. We only considered taxa that was present in at least in more than 15% of the samples. All multiple tests p-values were adjusted through the Bonferroni correction test. Although, since Bonferroni correction is a very conservative test, was also applied the false discovery rate using the Benjamini-Hochberg method.

4. Results

4.1 Sequencing data and taxonomic assignment

Before filtering, a total of 12,754,645 raw reads were obtained. The minimum number of reads per sample was 147 and the maximum was 1,085,170 reads, with a mean of 255,092.9 reads per sample. After the quality filtering, 752,666 reads remained but after removing those corresponding to mitochondria and chloroplast, 752,526 were used for further analyses. Three samples from diabetes patients did not pass the criteria due to the small number of reads, and therefore those samples were not included in further analyses. Nevertheless, without these samples, the variables age and sex remained not significantly different between the control and diabetes groups.

Diabetes patients group showed a higher average number of reads per individual (430,570) than the control group (321,956), but this difference is mostly due to a diabetic individual which demonstrates a large number of reads (Figure 1).

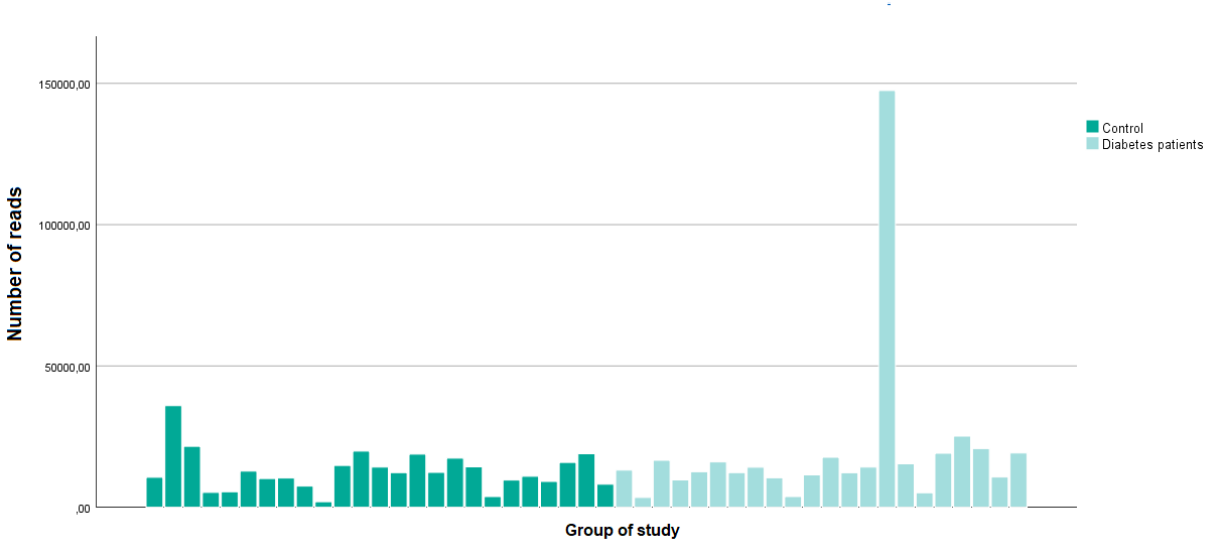

Figure 1. Number of reads per groups of study.

The remained high-quality reads were assigned to ASVs, which are amplicon sequences variants that differ by only one nucleotide. Considering the 47 samples there were in total 10,969 ASVs with a total absolute frequency of 752,526. The mean frequency of ASVs per sample was 15,053.32 (range: 1,973–147,455). After sample rarefaction, 92,731 reads (12.32%) and all 47 samples were retained in the data set.

After the alignment of the ASV to the oral microbiome database, the ASVs were assigned to 233 taxa. The taxa identification at the genus level was possible for 71% of the ASVs, and 21% at the species level. Control and diabetes groups shared 153 out of the 233 taxa. The control group exhibited 50 taxa that were not present in the diabetes group

and the diabetes group presented 31 taxa that were not in the control group. Nevertheless, each one of these not shared taxa were only present in one or two individuals. Furthermore, some of these taxa are disease-related still, its frequency is very low such as *Serratia marcescens*, which was only present in the control group (frequency of 3%).

At the phylum level, the oral cavity of all the 47 samples was dominated by *Firmicutes* (38%), *Proteobacteria* (30%), *Bacteroidetes* (18%), *Actinobacteria* (7%) and *Fusobacteria* (5%).

The twenty-four taxa that recruited 90% of the total reads is shown in Figure 2. *Streptococcus* genus was the most representative in terms of reads (26%), followed by *Gluconacetobacter* (18%) and *Prevotella* (11%).

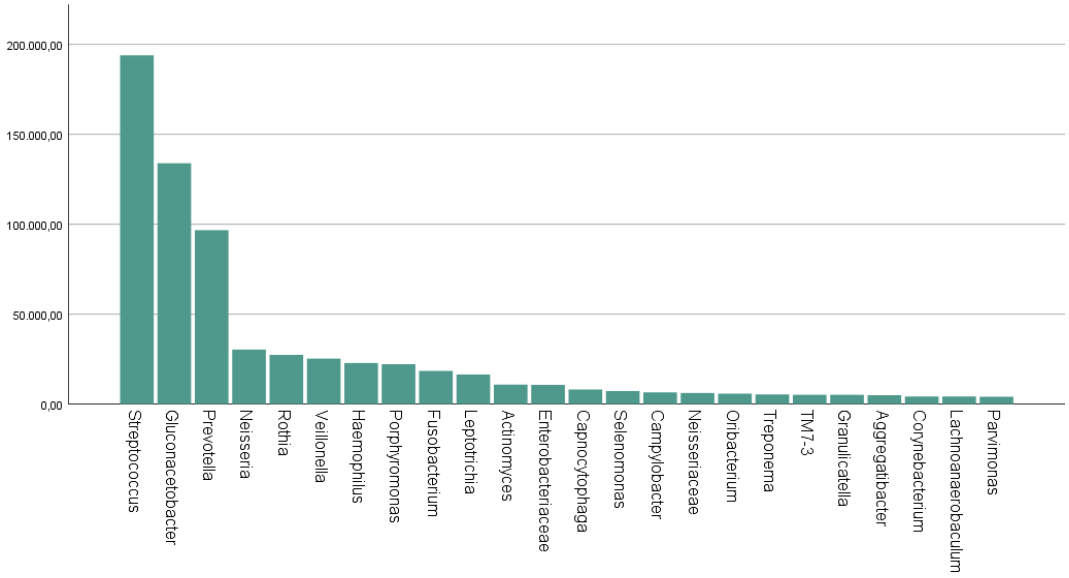

Figure 2. Taxa that recruited 90% of the total reads, up to the genus level.

4.2. Microbiome characterization

After sequence processing and alignment, it was possible to detect 12 phyla, 21 classes, 37 orders, 60 families, 86 genera and 51 species.

Taxa distribution for each individual at genus level is shown in Supplementary Figure 1. The mean number of taxa per individual is 54. For a better data visualization, the frequency distribution of the 10 most abundant taxa considering all samples, up to genera level is shown in Figure 3. *Streptococcus* is the most abundant taxa and is present in all subjects and *Gluconacetobacter* is highly present in one of the subjects, accounting for 90% of the total taxa in that individual.

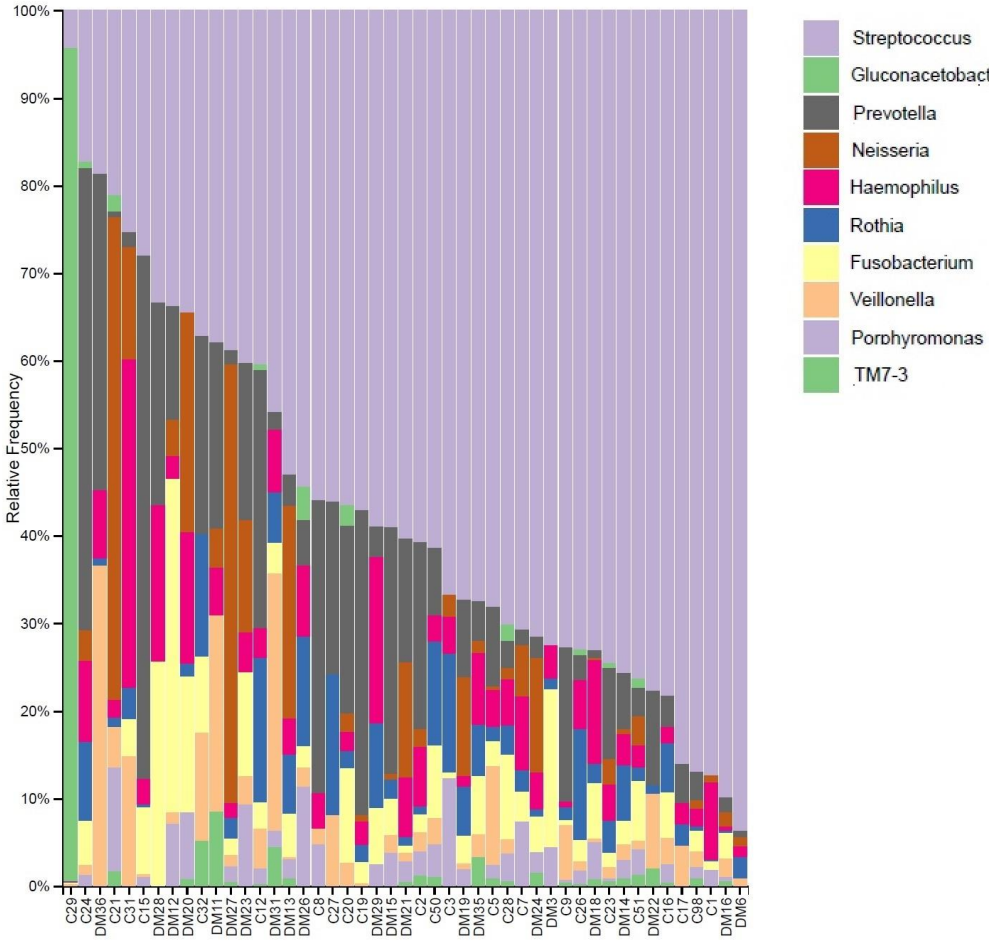

Figure 3. Ten most abundant taxa up to the genus level per subject. Sample names starting with C are from to the control group and those starting with DM are from the diabetics group.

Two heatmaps were built with taxa relative abundance. One with all the 233 taxa (Supplementary figure 2) and another with the taxa present in more than 15% of the samples (Figure 4). *Streptococcus* and *Prevotella* were the most abundant taxa, being *Streptococcus* present in all subjects with relative frequencies ranging (0.05-0.56). The remained taxa were present at lower frequencies. Considering these 10 most frequent taxa, the hierarchical clustering analysis formed two major clusters (Figure 4). These two clusters were formed due to *Streptococcus* frequency, where the first cluster aggregates the individuals with a high frequency of *Streptococcus* and the other cluster aggregates those with lower *Streptococcus* frequencies. Both clusters are composed of individuals from the control and diabetes groups.

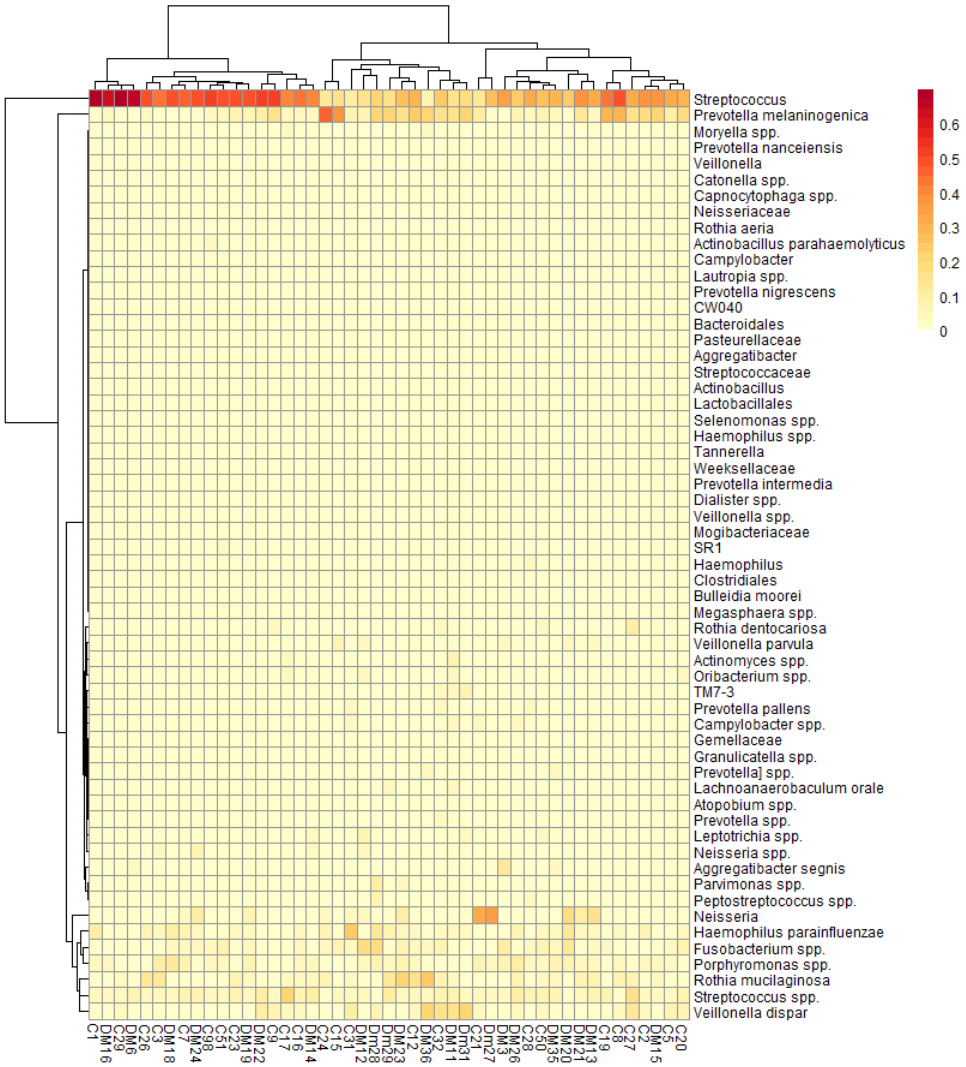

Figure 4. Heatmap of the relative abundance of the taxa that were present more than 15% of the samples. Each column of the heatmap represents the individuals from control (C) and diabetes (DM) groups and the rows are the taxa up to the species level. The vertical color bar on the right side of the graph defines the relative abundance of each taxon.

To evaluate the differences in microbial taxonomy between control and diabetes groups, the bacterial composition was compared at class, genus and specie levels with Mann-Whitney test. For statistical analyses all taxa were included, whereas in data visualization only the ten most abundant taxa of each group of this study were chosen.

Regarding the class level, only *Betaproteobacteria* (p=0.023), *Deltaproteobacteria* (p=0.015) and *Synergistia* (p=0.000) classes were significantly different between groups. The relative frequency of *Betaproteobacteria* was higher in the diabetes group and the other two classes were higher in the control group. However, when the Bonferroni test was applied, the *Synergistia* class was the only that remained significantly different between groups. Additionally, the classes *Saprospirae, Anaerolineae, Deltaproteobacteria* and *RF3* are only present in the control group.

The ten most frequent classes account for approximately 97% of the total abundance in both control and diabetes group (Figure 5). *Bacilli* and *Bacteroidia a*re the dominant classes in both groups, accounting for 63%. The main difference is that *Gammaproteobacteria* had a higher abundance in the control group (11.2%) than in the diabetes group (5.4%) whereas *Betaproteobacteria* was more abundant in the diabetes group (9.2%) when comparing to the control group (4.2%) (Figure 5).

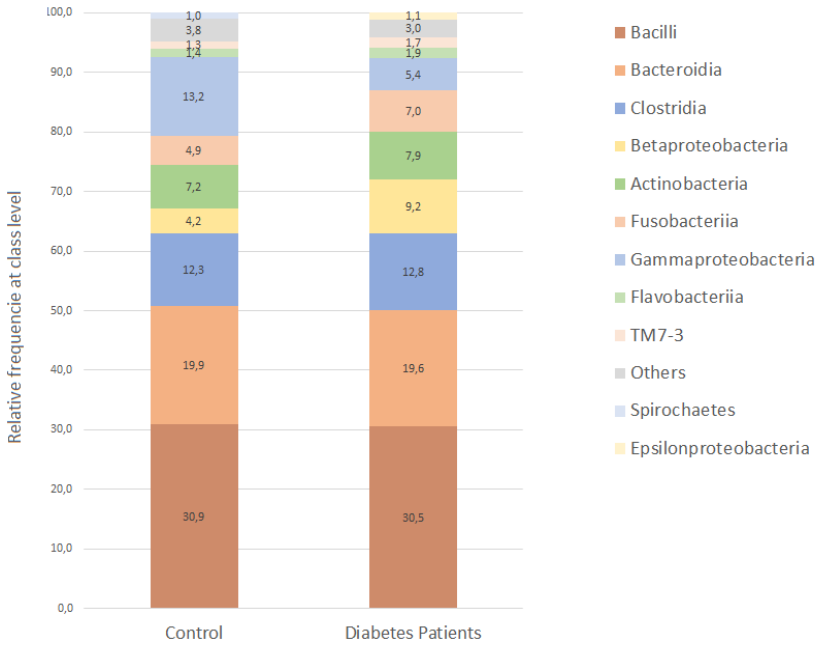

Figure 5. Relative abundance at class level of both control and diabetes groups.

When trying to obtain the taxa distributions at the genus level, for some of the taxa we were able to identify them only up to the family level due to lack of resolution. In any case, the frequency of the following taxa was statistically different between the control and diabetes groups: *TG5, Mycoplasma, Granulicatella, Acidaminobacteraceae, Filifactor, Mogibacteriaceae, Enterobacteriaceae, Gluconacetobacter, Neisseria, Tissierellaceae, Peptococcus, Porphyromonas, Bacteroidales, Moryella and Oribacterium* (p-value ranged from 0.000 up to 0.048). Nonetheless, when the Bonferroni correction was applied, only the *TG5* genus remained significant. It can be noted that *TG5* genus belongs to *Synergistia* class, the only class that was statistically significant.

The ten most-abundant taxa up to the genus level in each group represented approximately 77% in the control group and 78% in the diabetes group. *Streptococcus* and *Prevotella* were the most abundant taxa in both groups while *Actinomyces* and *Enterobacteriaceae* were the lowest in the control and diabetes groups, respectively. The major difference was the proportion of *Neisseria* abundance, which presence in the diabetes group was higher (7.3%) than in the control group (2.9%). The proportion of

Enterobacteriaceae and *Gluconacetobacter* taxa was also different between groups, which presence in the diabetes group was vestigial, 0.02% and 0.04% respectively (Figure 6 A).

The ten most-frequent taxa up to the species level from control and diabetes groups accounted for 64%-65% of the total taxa, respectively. *Streptococcus spp.* and *Prevotella melaninogenica* were the two most frequent species in both groups. Nonetheless, due to lack of resolution, only four of the 10 most abundant taxa, from each group, were assigned to their species level (Figure 6 B).

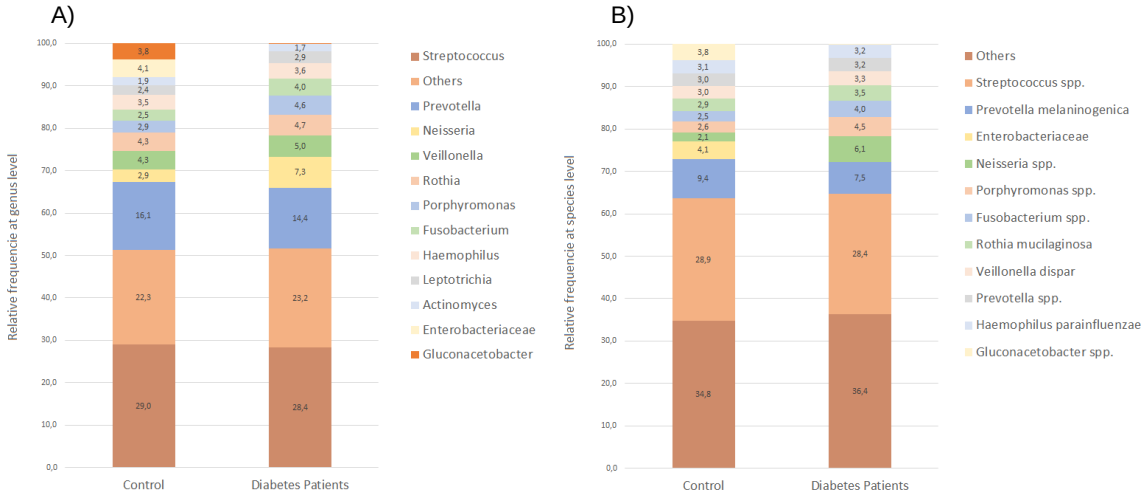

Figure 6. Relative abundance at the genus level (A) and at the species level (B) of both control and diabetes groups. Only the ten most-abundant taxa in each group are indicated. The remaining taxa are included in the category “Others”.

A total of 20 taxa, at the species level, were statistically significantly different between the two studied groups (note that due to the lack of resolution not all the taxa were identified up to species level): *TG5 spp., Mycoplasma spp., Granulicatella spp, Streptococcus anginosus, Acidaminobacteraceae*, *Bulleidia spp*. *Filifactor spp., Mogibacteriaceae, Enterobacteriaceae, Gluconacetobacter spp., Porphyromonas spp., Neisseria, Treponema socranskii, Rothia dentocariosa, Peptococcus spp., Tissierellaceae, Treponema spp., Bacteroidales, Moryella spp., Oribacterium spp* (p-value ranged from 0.000 up to 0.048). After Bonferroni correction, only TG5 spp. remained statistically significant, being more abundant in the control group.

4.3. Diversity measures

Before estimating the diversity measures it was necessary to select the sampling depth. Therefore, were performed rarefaction curves for the Shannon index and ASVs abundance, per individual. The rarefaction curves for the Shannon index showed that curves were close to a plateau over 1500 sequence reads (Figure 7) and the ASVs rarefaction curves progressively approached saturation, which reflects the species

richness (Figure 7). Hence, the samples were rarefied to 1973 reads, which surpasses the plateau value and it is also the lowest value in the dataset, to keep the maximum number of samples and ASVs as possible

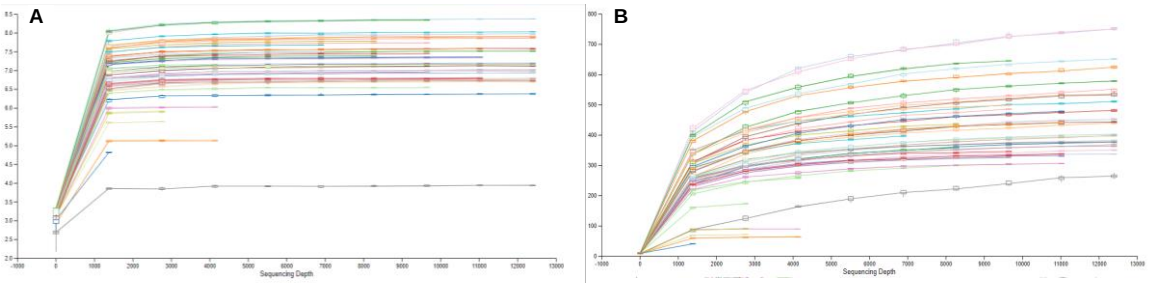

Figure 7. Rarefaction curves from (A) the Shannon index and (B) the ASVs abundance per individual.

As a measure of alpha diversity (diversity per individual) we used the Shannon index and the ASVs abundance, which were compared between control/diabetes groups, as well as between different categories of the following variables: sex, BMI, age, macronutrients, sugar, coffee, alcohol consumption, energy intake and hygiene habits (teeth brushing and mouthwash use). Variables such as age, macronutrients, sugar, coffee, alcohol and energy intake, were divided into three categories according to percentiles (33% percentile, 33-67% percentiles, and 67-100% percentiles).

While Shannon index distribution is practically the same in both groups (Figure 8), in the ASVs observation, the control group shows a higher count of ASVs (Figure 9). However, the distribution of the Shannon diversity values was not significantly different between the control and diabetes groups (p=0.831), nor for the ASVs abundance (p=0.216).

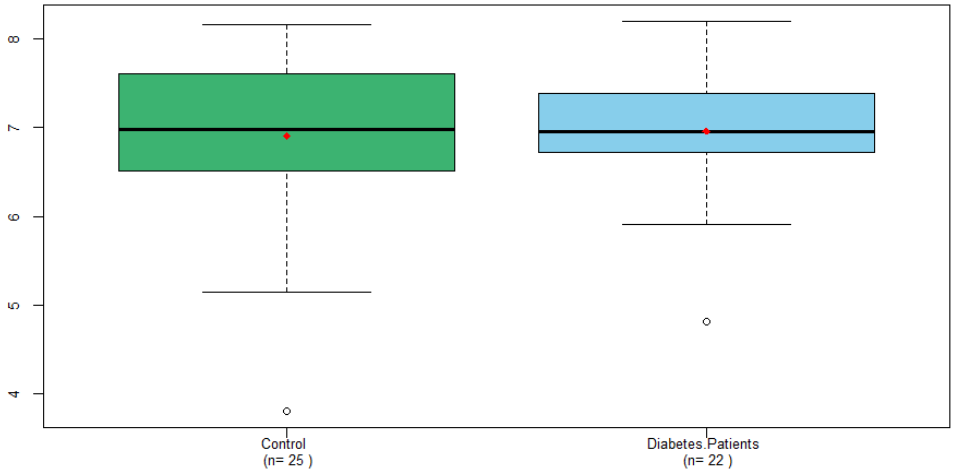

Figure 8. Boxplot charts depicting the distribution of the Shannon index in both groups; Red dot represents the mean of each group.

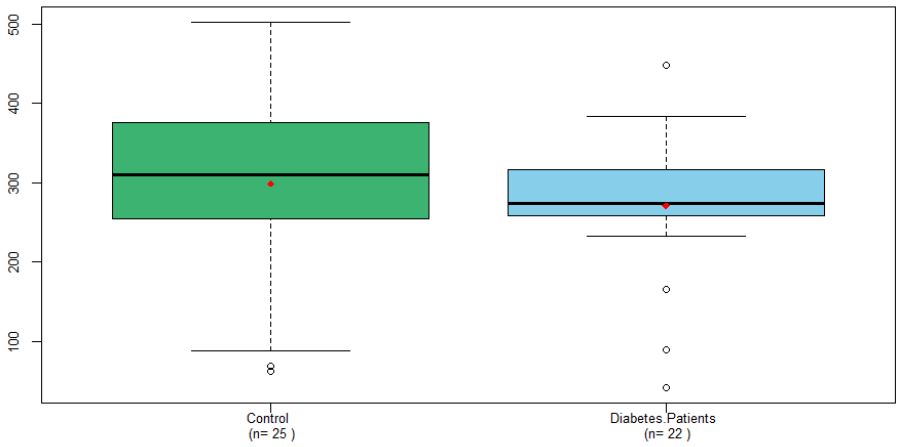

Figure 9. Boxplot charts depicting the distribution of the ASVs abundance per individual in both groups; Red dot represent the mean for each group.

Concerning the sex variable, the Shannon index and ASVs abundance were calculated first with both groups together and then separately according to the group of study.

Males showed a smaller Shannon index (Figure 10) and less ASVs count than females (Figure 11), but none of these comparisons were statistically significant (p=0.553; p=0.398, respectively). Likewise, none of the comparisons were statistically significant when sex was divided according to the group.

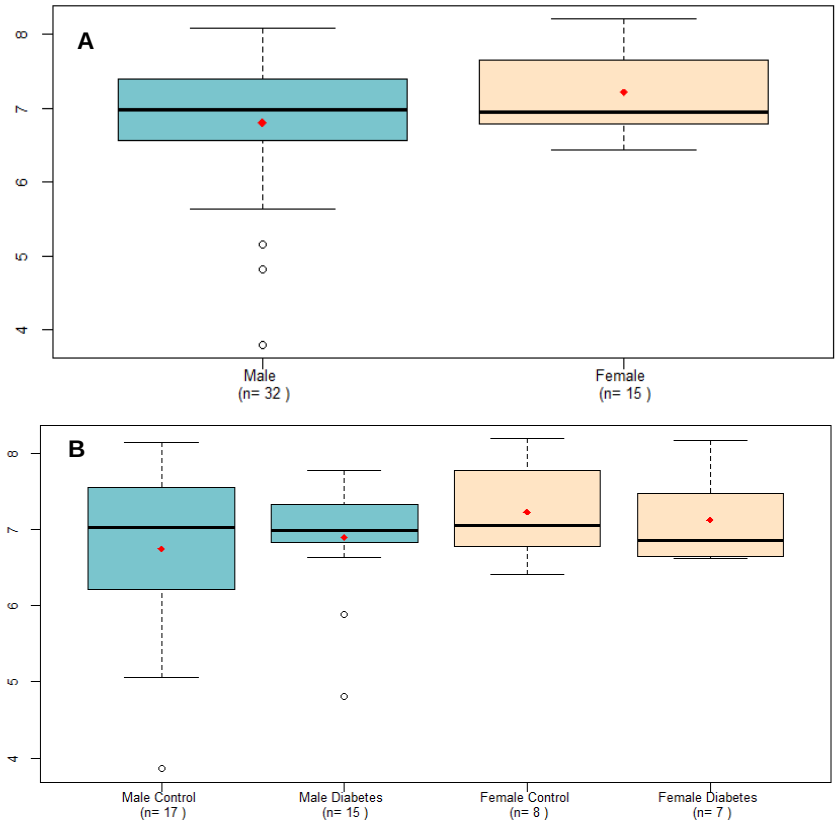

Figure 10. Boxplot charts depicting the distribution of the Shannon index for (A) both sexes, and (B) according to sex and group of study. Red dot represents the mean of each group.

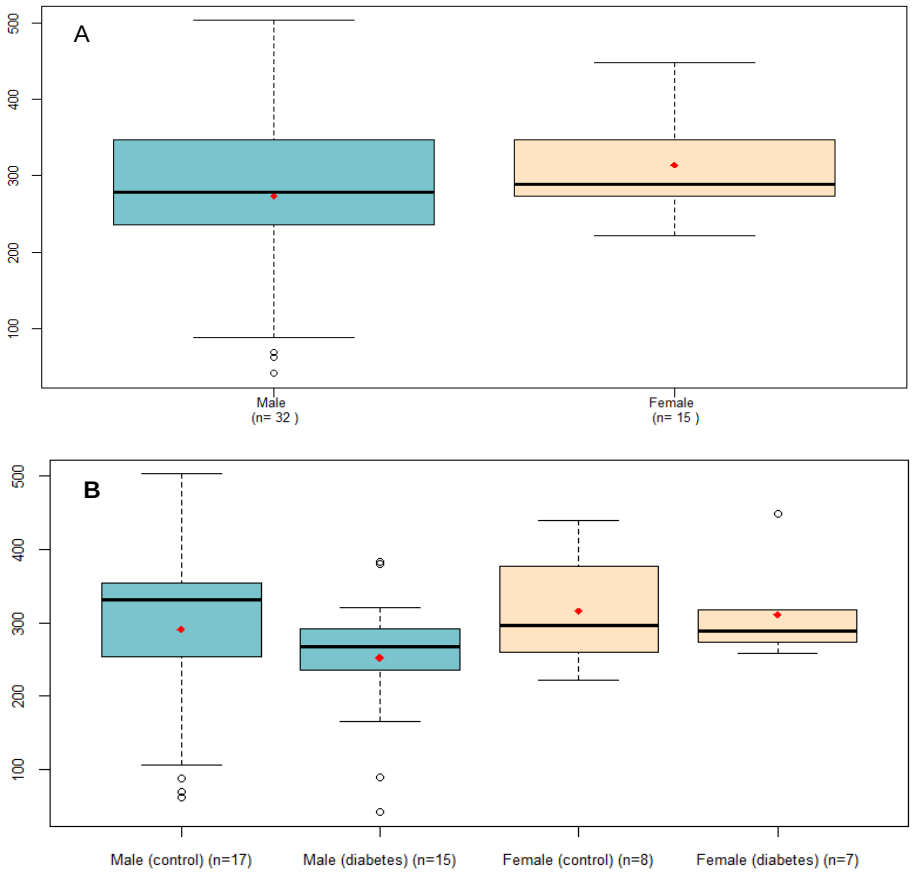

Figure 11. Boxplot charts depicting the distribution of the ASVs abundance for (A) both sexes, and (B) according to sex and group of study. Red dot represents the mean of each group.

As done in the sex variable, we calculated the alpha diversity measures grouping the individuals by BMI categories, considering the control and diabetes subjects together and with both groups separated.

When we considered all individuals together, the obese class III showed a Shannon index higher than the rest of the categories, and the normal weight category showed the lowest. In general, individuals with overweight revealed a higher index (μ=7.2) than the normal weight (μ=6.5; Figure 12). Observed ASVs shown the same tendency than the Shannon index (Figure 13), and none of the comparisons between categories were statistically different.

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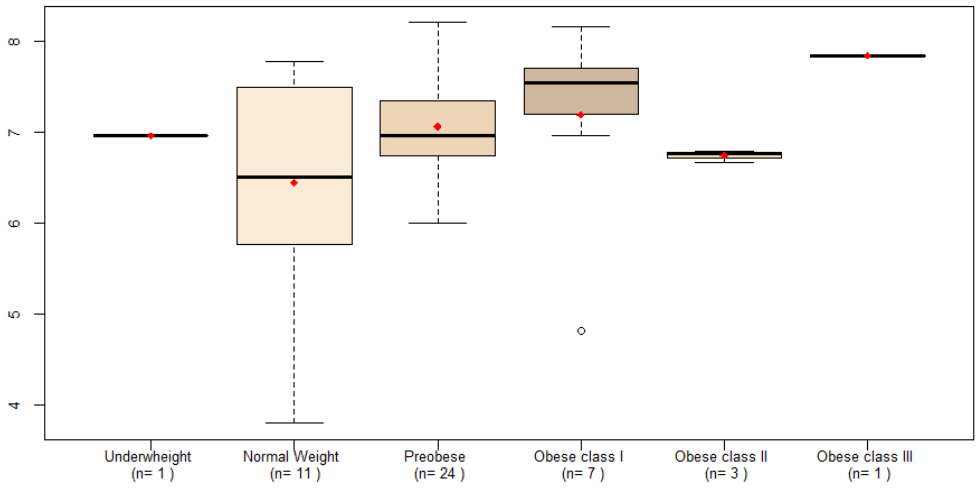

Figure 12. Boxplot charts depicting Shannon index distributions according to BMI categories. The red color dots represent the mean of each category.

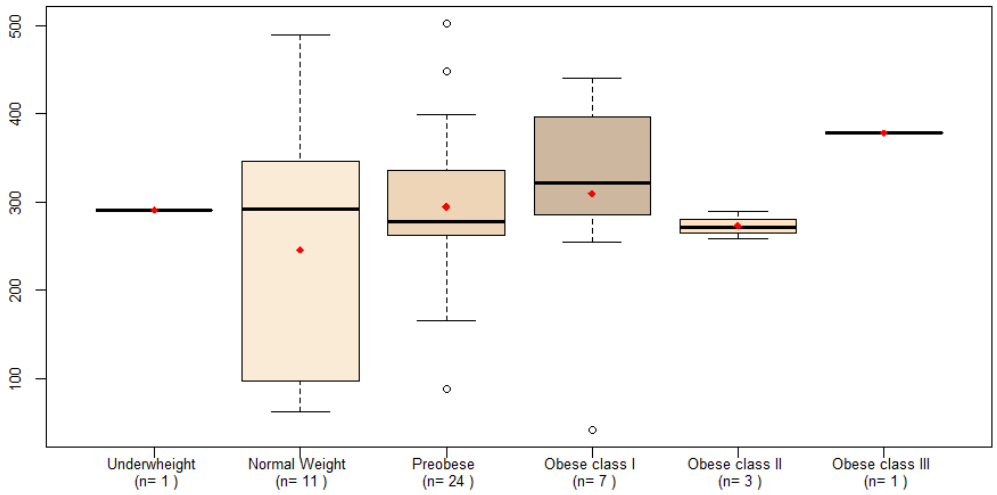

Figure 13. Boxplot charts depicting ASVs abundance distributions according BMI categories. The red color dots represent the mean of each group.

When control and diabetes groups were separated according to BMI, the obese class III and the normal weight, from the control group, showed the highest and the lowest Shannon index respectively (Figure 14). Regarding diabetes group, pre-obese group and obese class I showed highest and the lowest Shannon index correspondingly (Figure 14).

ASVs abundance showed the same tendency as Shannon index distribution (Figure 15). None of the comparisons of these distributions were significantly different.

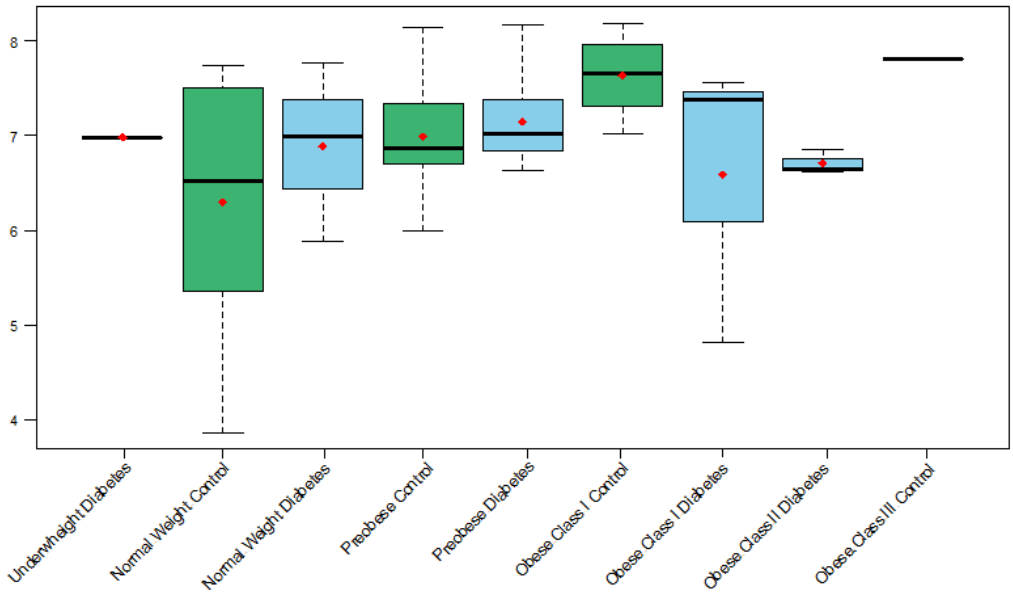

Figure 14. Boxplot charts depicting the Shannon Index distribution according to the group of study and BMI. The red color dots represent the mean of each category.

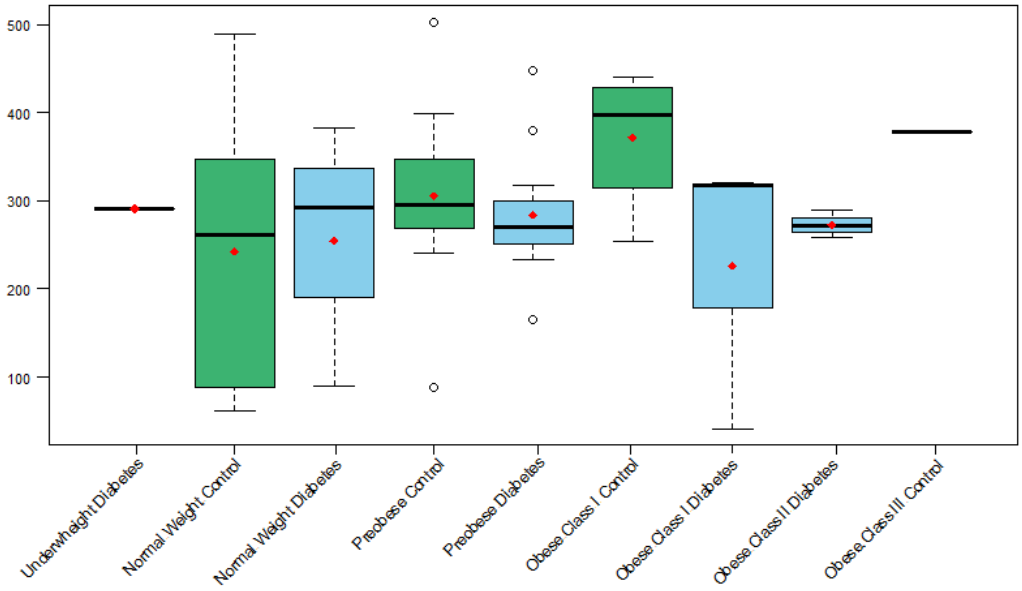

Figure 15. Boxplot charts depicting the ASVs abundance distribution according to the group of study and BMI. The red color dots represent the mean of each category.

Regarding the distribution of the Shannon index, when age, carbohydrates, coffee, total fat, and energy intake variables were considered, the category 2 (percentiles 33%-67%) presented the highest index, followed by category 3 and 1 (Supplementary figure 3-7).

Shannon index distributions regarding sugar and protein consumption showed that the category 3 (percentiles 66-100%) had the highest values, followed by category 2

(percentiles 33% - 67%) and 1 (percentile 0-33%), indicating that the more the amount of sugar and protein consumption, the higher the index (Supplementary figure 8-9).

Category 1 (percentile 0-33%) from alcohol consumption presented the highest Shannon index, followed by category 2 (percentiles 33-67%) and 3 (percentiles 67-100%). This indicates that the Shannon index appears to decrease with alcohol consumption (Supplementary figure 10).

The results when we considered hygiene habits such as teeth brushing, showed that those who wash their teeth once at three times per week have a higher Shannon index followed by those who wash once per day and more than once per day. Those that never wash their teeth showed the lowest index value (Supplementary figure 11). Additionally, increasing the use of mouthwash seems to increase the Shannon index (Supplementary figure 12).

Finally, smoking habits were divided into heavy smokers, moderate smokers and non-smokers. Heavy smokers showed a higher index whereas moderate smokers presented the lowest index value (Supplementary figure 13).

None of the comparisons between the above-mentioned categories were statistically different. The ASVs count followed the same tendency (Supplementary figure 14–23) as the Shannon index, except in alcohol consumption where category 3 presented the highest ASVs count and category 1 the lowest (Supplementary figure 24).

4.3.2. Beta diversity

Bray Curtis dissimilarity distances were calculated to measure the differences between individuals, in terms of taxonomic structure. Figure 16 shows the beta diversity values between individuals belonging to the same group of study and between individuals from the two groups.

The beta diversity values within each of the groups were similar to the distances between the two groups (pseudo-F=1.039; p=0.322) (Figure 16), indicating that there is no differentiation in the microbiome composition of the diabetes group compared to the control one.

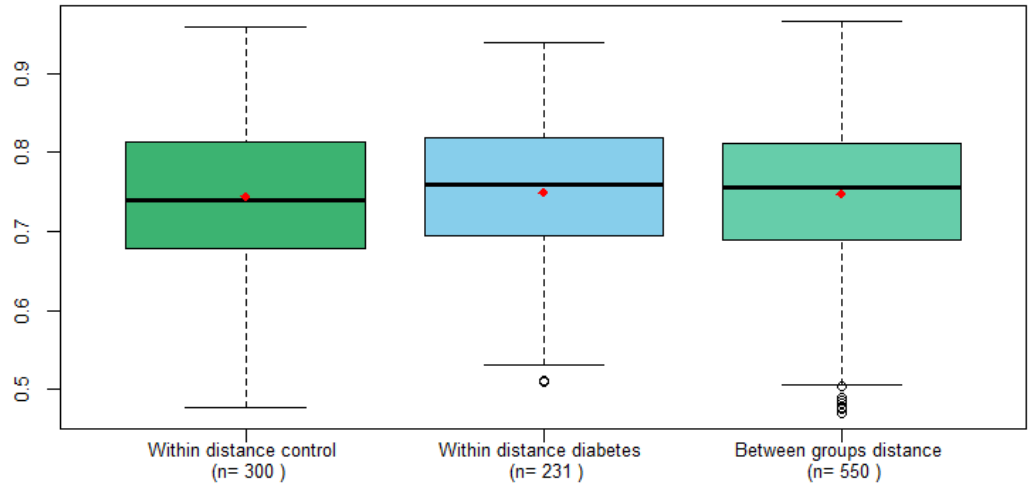

Figure 16. Bray Curtis dissimilarity values within each of the two groups studied and between them. The red color dots represent the mean of each group.

The beta diversity distances between individuals belonging to the same sex were similar to the distances obtained between individuals of different sex (pseudo-F=1.159; p=0.166) (Figure 17). Therefore, no difference in the microbiome structure was found according to sex.

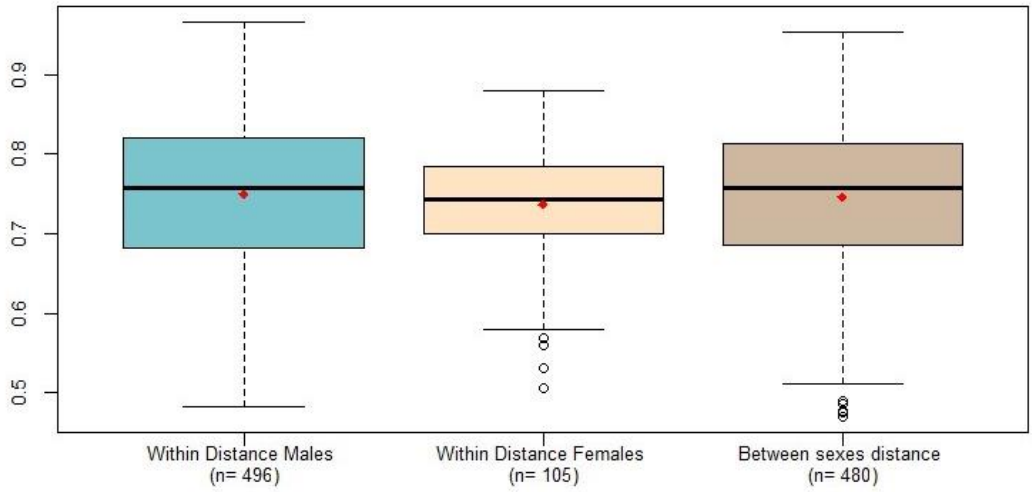

Figure 17. Bray Curtis dissimilarity values according to sex. The red color dots represent the mean of each group.

To better visualize whether existed a taxonomic structure in the samples analyzed we performed a Principal coordinates analyses (PCA) plot from the Bray Curtis dissimilarity matrix. The PCA plot did not reveal a clear clustering pattern, being all individuals from the control and diabetic patient groups scattered throughout the plot. Also, when other variables were tested such as hygiene, smoking and dietary habits (not shown) the PCA plot still did not show a clear clustering pattern. However, it is possible to observe one major aggregation and some outside groups (Figure 18).

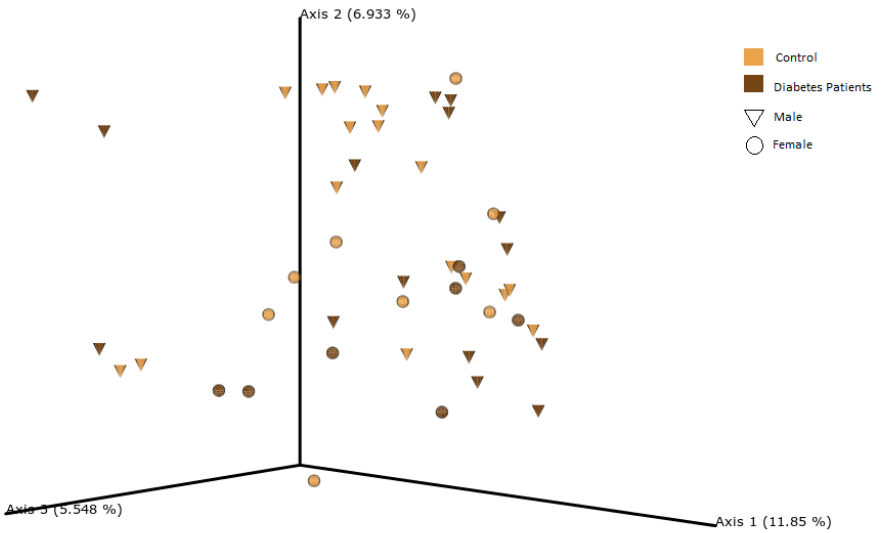

Figure 18. PCA plot showing the first, second and third principal components and the percentage of the total variance that they explain based on Bray Curtis dissimilarity matrix. Each point represents one individual, which color and symbol indicate the group of study and sex.

4.4. Bacteria associated with periodontal disease

As some studies showed that individuals with diabetes have a higher predisposition to periodontitis, we investigated the relative frequency of the bacteria associated with periodontal disease in our samples. The species that were found to be related to this disease were *Prevotella intermedia, Campylobacter rectus, Porphyromonas endodontalis,* and *Treponema socranskii.* Although the relative abundance of these species was almost vestigial in both groups, all of these taxa were more frequent in the control group. Additionally, only *Treponema socranskii* was statistically different between groups (p=0.026). Nevertheless, when the Bonferroni correction was applied, this difference was not significant.

4.5. Diet

The dietary frequency questionnaire applied to the individuals from both groups provided the daily intake of 91 nutrients and energy intake. From the 91 nutrients, only the macronutrients are presented, including also BMI, sugars, monounsaturated, polyunsaturated and saturated fats variables (Tables 1-3). For each of those variables mentioning before, their distributions between the two studied groups were compared through Mann-Whitney U test or Student's T-test (when the variable was normally distributed), with a 5% level of significance.

The diabetes patients had a higher energy intake, BMI, and intake of nutrients, except for sugar consumption, than the control group (Table 1). Protein consumption was the

only variable that presented a significant difference between both groups, the diabetes patients group showed a higher protein intake than control group (Table 1). However, when the Bonferroni correction was applied, this difference was not significant (p=0.387).

Table 1. Descriptive statistics of nutrients consumption, energy intake and BMI of both control and diabetes groups and respective p-values of Mann-Whitney test/Student's T-test between the same groups.

	Control	Diabetes Patients	
	Mean ± SD	Mean ± SD	p-value
Protein	94.5 g/day ± 32.2	111.1 g/day ± 35.4	0.043*
Carbohydrates	218.5 g/day ± 70.9	236.2 g/day ± 75.0	0.839
Sugar [t]	92.5 g/day ± 31.5	84.0 g/day ± 32.6	0.352
Total fat	73.4 g/day ± 27.2	85.0 g/day ± 35.8	0.432
Monounsaturated fat	34.4 g/day ± 13.2	40.4 g/day ± 19.0	0.421
Polyunsaturated fat	12.4 g/day ± 4.7	15.3 g/day ± 7.5	0.607
Saturated fat	20.4 g/day ± 8.7	21.9 g/day ± 8.0	0.421
Calories	1946.7 Kcal/day ± 584.1	2199.8 Kcal/day ± 726.4	0.594
Body Mass Index	26.9 Kg/m² ± 4.6	28.4 Kg/m² ± 5.2	0.728

* Statistically significant

[t] - Variables to which Student's T-test was applied. The remaining variables were analyzed using Mann-Whitney test.

For each group, a comparison of nutrients consumption, energy intake and BMI were also performed according to sex (Table 2 and 3).

Regarding the control group, women had a higher consumption of nutrients, energy intake and BMI than men. Total fat and polyunsaturated fat were the only variables where the difference between sexes was significant (Table 2). Nevertheless, when the Bonferroni correction was applied, these differences were not significant (p=0.369; p=0.117).

Table 2. Descriptive statistics of nutrients consumption, energy intake and BMI of the control group, according to sex and respective p-values of the comparisons between sex, through Mann-Whitney and Student's T tests.

	Control		
	Male	Female	
	Mean ± SD	Mean ± SD	P-value
Protein	85.0 g/day ± 20.4	114.7 g/day ± 43.8	0.157
Carbohydrates	210.6 g/day ± 69.0	235.3 g/day ± 76.7	0.669
Sugar	87.1 g/day ± 7.2	103.9 g/day ± 12.0	0.219
Total fat [t]	65.9 g/day ± 21.1	89.4 g/day ± 33.0	0.041*
Monounsaturated fat [t]	31.0 g/day ± 11.0	41.6 g/day ± 15.5	0.590
Polyunsaturated fat [t]	10.8 g/day ± 3.4	15.7 g/day ± 5.7	0.013*
Saturated fat	18.4 g/day ± 7.2	24.7 g/day ± 10.5	0.100
Calories [t]	1832.6 Kcal/day ± 483.3	2188.3 Kcal/day ± 733.4	0.160
Body Mass Index	25.9 Kg/m² ± 3.4	29.0 Kg/m² ± 6.2	0.238

* Statistical significant

[t] - Variables to which Student's T-test was applied. The remaining variables were conducted with the Mann-Whitney test.

In the group of the diabetes patients, the nutrients consumption was higher in the female group, except in the case of protein intake. Energy intake and BMI were also greater in women, being the BMI statistically different between sexes (Table 3). Through Bonferroni correction, BMI lost its statistical significance (p=0.108).

Table 3. Descriptive statistics of nutrients consumption, energy intake, and BMI of diabetes group, according to sex and respective p-values of the comparisons between sex, through Mann-Whitney and/or Student's T tests.

	Diabetes Patients		
	Male	Female	
	Mean ± SD	Mean ± SD	P-value
Protein	113.2 g/day ± 34.0	106.7 g/day ± 40.3	0.750
Carbohydrates [t]	232.9 g/day ± 69.5	243.0 g/day ± 90.3	0.761
Sugar	80.9 g/day ± 7.6	90.7 g/day ± 12.8	0.588
Total fat	80.0 g/day ± 27.1	95.6 g/day ± 50.2	0.932
Monounsaturated fat	37.4 g/day ± 14.2	46.6 g/day ± 26.7	0.711
Polyunsaturated fat	14.3 g/day ± 6.2	17.3 g/day ± 9.9	0.932
Saturated fat	21.1 g/day ± 6.2	23.5 g/day ± 11.3	1.00
Calories	2173.0 Kcal/day ±624.9	2256.8 Kcal/day ± 954.6	0.669
Body Mass Index	26.3 Kg/m² ± 3.5	32.9 Kg/m² ± 5.6	0.012*

* Statistically significant

[t] - Variables to which Student's T-test was applied. The remaining variables were conducted with the Mann-Whitney test.

For each variable, the respective values are presented according to its group of study (Figure 19-21 and Supplementary figures 25-31) and sex (Supplementary Figures 32-37) to evaluate the data distribution, except for the BMI variable, which was presented with a bar plot (Figure 22). Only those variables that were statistically different between groups are shown in the results, being the rest in the supplementary material.

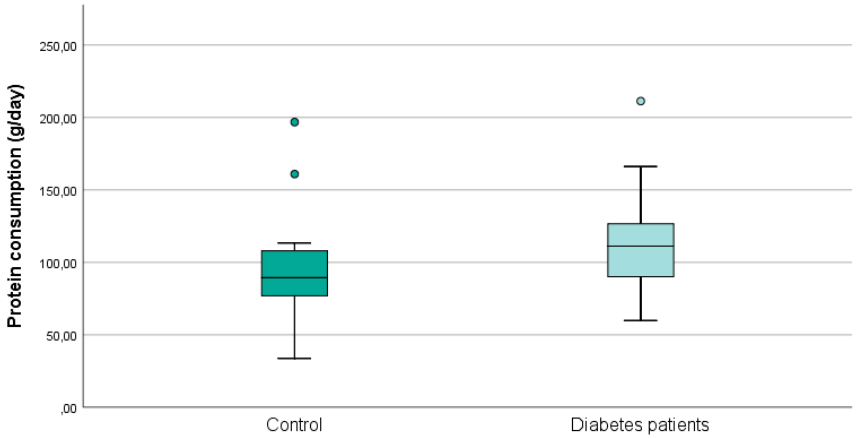

Figure 19. Protein consumption (g/day) in both diabetes and control groups.

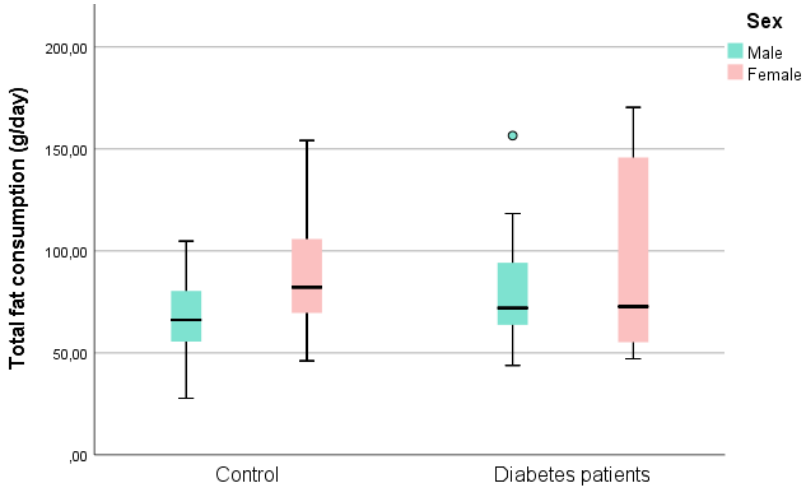

Figure 20. Total fat consumption (g/day) in both diabetes and control groups, according to the sex.

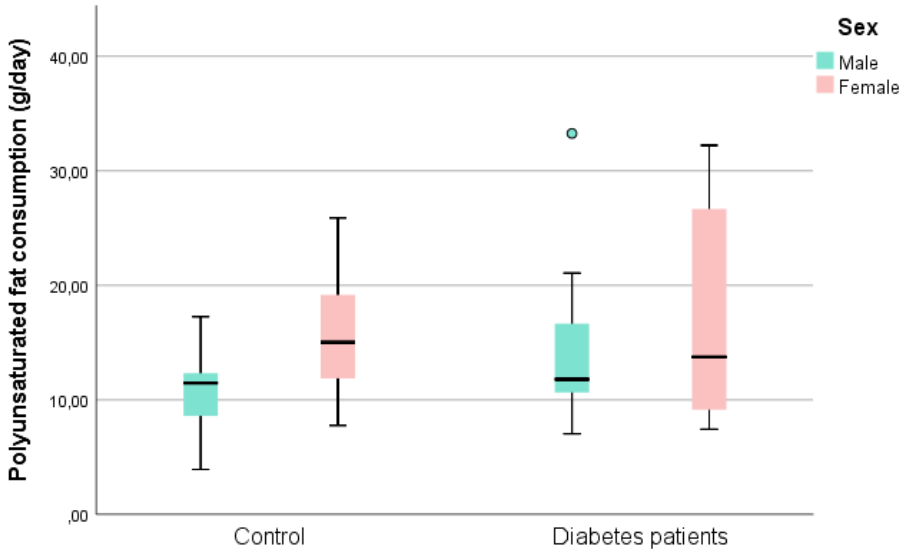

Figure 21. Polyunsaturated fat consumption (g/day) in both diabetes and control group, according to the sex.

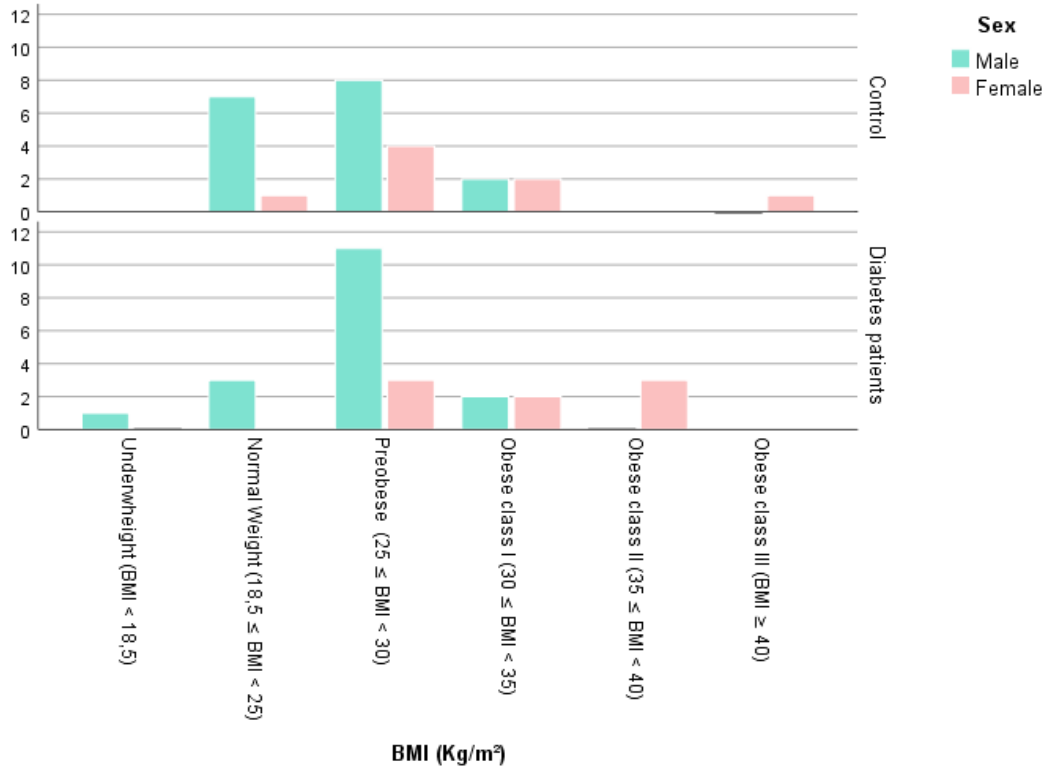

Figure 22. Body Mass Index (Kg/m²) of diabetes and control groups according to the sex.

4.6. Correlation between diet and microbiome

To investigate if there was association between the diet and the oral microbiome, we carried out Spearman correlation tests between each of the 91 diet variables and each taxa of the oral microbiome. When Bonferroni test was applied, none of the correlations remained significant. As the Bonferroni test is very conservative, a false discovery rate through the Benjamini-Hochberg method was also applied. However, with this method

also none of the correlations remained statistically significant. Therefore, only those with a p-value lower than 0.001, before the corrections tests and those with correlations to macronutrients, polyunsaturated, saturated fat and sugars are described.

Porphyromonas presented positive correlations with butyric (ρ=0.494; P<0.000), caproic (ρ=0.509; P<0.000) and lauric acids intake (ρ=0.500; P<0.000). *Rothia mucilaginosa* showed positive associations with arachidic acid (ρ=0.535; P<0.000) and behenate (ρ=0.494; P<0.000). *Haemophilus* correlates negatively with myristoleic acid (ρ=-0.515; P<0.000) and *Campylobacter* obtained positive correlations with butyric (ρ=0.577; P<0.000), caproic (ρ=0.528; P<0.000), caprilic (ρ=0.562; P<0.000), capric (ρ=0.530; P<0.000), lauric (ρ=0.514; P<0.000) and myristic acids intake (ρ=0.537; P<0.000).

Regarding the macronutrients, *Rothia dentocariosa*, *Rothia mucilaginosa species,* and *Streptococcaceae* family, showed a correlation with protein intake, whereas for *Rothia dentocariosa* there was a negative correlation (ρ=-0.297; P=0.043), for the other two taxa the correlation was positive (ρ=0.297; P=0.043; ρ=0.310; P=0.034).

Rothia mucilaginosa was the only taxa positively correlated with carbohydrates (ρ=0.409; P=0.004), polyunsaturated fat (ρ=0.421; P=0.003), and total fat (ρ=0.310; P=0.034).

Campylobacter, Streptococcaceae and *Haemophilus* were positively correlated to saturated fat (ρ=0.339; P=0.020; ρ=0.321; P=0.028; ρ=0.291; P=0.047) and F16 family was the only taxa associated to sugar intake (ρ=0.317; P=0.030)

Finally, *Rothia mucilaginosa* and *Veillonella* were the only taxa related to complex carbohydrates intake (ρ=0.307; P=0.036; ρ=0.318; P=0.029).

5. Discussion

This study aimed to investigate the oral microbiota in diabetic individuals from Portugal using the 16S rRNA sequencing method and to investigate the diet influence in the oral microbiota. The group of diabetic individuals was compared to a non-diabetic control group.

Overall, the control group showed a higher amount of taxa (202 taxa) than the diabetes group (183 taxa) which is in line with previous studies (e.g., Saeb et al., 2019). Also, among those 81 not shared taxa, some are disease-related such as *Serratia marcescens,* which is an opportunistic pathogen related to respiratory and urinary infections. This species has also been associated with eye infections, endocarditis, septicemia, and osteomyelitis.

Through taxonomic assignment, it was possible to infer that at the phylum level, the oral microbiome of both control and diabetes groups was dominated by *Firmicutes* (38%), *Proteobacteria* (30%), *Bacteroidetes* (18%), *Actinobacteria* (7%) and *Fusobacteria* (5%), constituting 98% of the total oral microbiome. Previous studies also reported these phyla as the most abundant ones, in the oral cavity (Aas et al., 2005; Verma et al., 2018; Yang et al., 2012). Furthermore, *Actinobacteria* was found to be more abundant in the control group, which is in line with Long et al. (2017) findings, in which *Actinobacteria* was significantly associated with a decreased risk of diabetes.

At the class level, the most abundant taxa in both groups were *Bacilli* and *Bacterioidia*. The main difference between the two groups was *Gammaproteobacteria* and *Betaproteobacteria* abundance, being the most abundant class in the control group and the second most abundant class in the diabetes group. Saeb et al. (2019) compared the oral microbiome between normal glycemic and type 2 diabetic individuals and also reported *Bacilli* and *Bacterioidia* as the most abundant classes, being both classes more abundant in the normal glycemic group. *Gammaproteobacteria* abundance was also higher in the control group and the *Betaproteobacteria* richness higher in the diabetes group, which is in line with our findings. Nevertheless, these differences were more pronounced in our results.

At the genus level, the most abundant taxa were *Streptococcus* and *Prevotella* followed by *Neisseria* in the diabetes group and *Veillonella* in the control group. A study that analyzed the oral microbiota of healthy adults reported that *Prevotella, Streptococcus, and Veillonella* genera were responsible for about 50% of the total salivary microbiome (Keijser et al., 2008), which is similar to our findings. When comparing to Saeb et al.

(2019) study, the predominant genus of the control group was *Prevotella* followed by S*treptococcus,* which is not in accordance with our results.

As it was not possible to attain the species-level identification to all taxa, an approach to increase this resolution could be the use of the shotgun sequencing method as a complement of the technique here used.

In regard to the alpha diversity values, the ASVs abundance was higher in the control group however the Shannon index was similar in both groups. While ASVs abundance reflects the microorganisms' richness, the Shannon index also expresses the evenness. So, our results indicate that the diabetes group had a lower richness of taxa, but with a more even distribution. Some studies reported that diabetes patients have less diversity than the control group (Sabharwal et al., 2019) while other studies reported the opposite (Casarin et al. (2013). However, in Casarin et al. (2013), the diabetes patients were not controlled by medication, a factor that could, indeed, explain these differences. In our study, some variables could explain the lack of differences between the control group and the diabetes patients, such as the low number of samples, which lowers the statistical power, and that the diabetes patient's medication can potentially interfere with the oral microbiome. Additionally, we need to take into account that some data about personal and dietary habits could not be completely accurate due to the subject omission by embarrassments such as not having healthy oral and dietary practices or a distorted perception of the food quantities that they ingest. Furthermore, since biological, genetic and environmental factors influence the oral microbiome, a comparison between the oral microbiome from the general Portuguese population provided in this work with other populations, with the same age category, could help to better understand these influencing factors.

As far as sex, women showed higher diversity (control: 316; diabetes: 311) than men according to the number of ASVs (control: 291; diabetes: 252) in both groups. One study reported that males had a higher alpha-diversity than females yet, this study explored the oral microbiota in children and not in adults (Raju et al., 2019). Studies that investigated the oral microbiome according to sex are scarce and mainly focused on the gut microbiome. However, studies on gut microbiome associated with sex showed inconsistent results (Mueller et al., 2006; Takagi et al., 2019) and were mainly focused on taxonomic composition, without measuring alpha and beta diversity between sexes. Also, it is worth mentioning that the number of females in our study was lower than the number of males, which could have influenced our results.

On the subject of the analysis relating the BMI and the oral microbiome, although there were no significant differences according to BMI, subjects overweight showed an increased the Shannon index than the normal weight ones which is not in accordance with the studies of Wu et al. (2018) and Tam et al. (2018). These authors found the Shannon index to be lower in the obesity group when compared to the normal-weight group.

Other factors can influence microbiome abundance and composition. For instance, we could not find a clear pattern of the alpha diversity analyses of age, carbohydrates, coffee, total fat, and calories intake. Although, there a large plethora of studies regarding the association between the gut microbiome and the aging process, the influence of this on the oral microbiome composition is poorly documented. Indeed, from those studies, some suggested that microbiome does not change in elders, and others reported that with age the microbiome diversity reduces (Biagi et al., 2010). Since the age variation of the individuals from both groups was small, and most of them fit in the elderly category, it was not possible to conduct comparisons. That is, to compare microbiome diversity according to age evolution it would be necessary to perform more studies on younger individuals from Portugal. Higher intake of carbohydrates has been correlated to periodontitis (Hujoel, 2009) and consequently to the loss of diversity (Jorth et al., 2014). However, in the present study, those that had a higher carbohydrates intake did not show a less diverse microbiome than those who had fewer carbohydrates consumption. The coffee influence in the oral microbiome was also not conclusive. Some studies did not find any relation between coffee and microbiome diversity (Peters et al., 2018) while others reported that coffee was associated with a reduced oral microbiome diversity (Signoretto et al., 2010). Studies about how dietary fat affects the microbiome are lacking, especially regarding oral microbiome. Interestingly, gut microbiome studies have reported contradictory results, some suggesting that a high-fat diet decreases the diversity and richness of the microbiome (Zhang et al., 2012), whereas others stated the opposite (Shortt et al., 2018). There are no studies respecting calories and microbiome diversity however, the main influencers of diet in the microbiome are nutrients in general and not calories.

Our analysis revealed that higher amounts of protein and sugar intake were related to higher Shannon index and richness. Studies about oral diversity according to protein and sugars are scarce because the focus is usually on microbial composition and not in alpha and beta diversity. A study comparing the oral microbiome between hunter-gatherers and agricultural populations, found a higher microbiome richness in the first group. The authors suggested that this difference was due to diet differences, where the hunter-

gatherers would have a higher intake of protein (Nasidze et al., 2011), which is in line with our findings. Concerning sugar intake, we expected a smaller alpha index in those with higher intake, following the arguments on carbohydrates consumption, however, our findings showed a slightly opposite tendency.

Individuals presenting greater alcohol intake showed a slightly small Shannon index, which contradicts previous findings. Fan et al. (2018) revealed a higher diversity in heavy and moderate drinkers than in non-drinkers although the authors suggest that could be due to the direct effects of the alcohol or due to poor oral health conditions in drinkers.

It is known that the oral microbiome diversity decreases with good oral hygiene habits. However, studies focused on the impact of teeth brushing in the oral microbiome usually report what type of bacteria exists according to the level of hygiene, but without providing diversity measures. According to our analysis, those who wash their teeth once at three different times per week have a higher diversity than those who wash every day or do not wash them. It could be expected that those without oral care would show a more diverse microbiome if there were no signs of oral diseases, as periodontitis reduces oral diversity. Also, the higher diversity observed in those who only wash their teeth three times per week could be due to a higher accumulation of oral bacteria in the mouth than those who wash them every day. Most of mouthwashes present antimicrobial properties so, it would be expected a decrease in biodiversity with the increase of mouthwash use (Okuda et al., 1998; Tribble et al., 2019); however, our data reported a slightly opposite tendency.

Yet, another practice that affects the oral microbiome composition is smoking. In our findings, heavy smokers presented a higher diversity index, which is in agreement with previous studies that reported that smokers tend to have a more diverse microbiota, including pathogenic taxa than non-smokers (Mason et al., 2015; Takeshita et al., 2016).

As far as the beta diversity, no significant differences were found between groups. Similar groups have smaller distances from each other and if there was a difference, it would be expected that the between-group distance to be higher than the within-group distances. However, our between-group distances did not differ from within-group distances ($p=0.166$), and therefore there was no difference in microbial composition between control and diabetes groups. This result was also consubstantiated by the PCA analysis where no clustering of the samples according to the two groups studied was observed.

Several studies have related diabetes type 2 to periodontitis prevalence and incidence (Preshaw et al., 2012). There, the species found to be related to periodontitis were *Prevotella intermedia, Campylobacter rectus, Porphyromonas endodontalis,* and *Treponema socranskii.* Nevertheless, these species presented a higher relative frequency in the control group.

The second part of this study aimed to elucidate the association between diet and microbiome. According to our results, certain dietary components showed associations with the oral microbiome, especially medium-chain fatty acids (caprylic, capric, and lauric acid), saturated fatty acids (SFAs) (behenate, arachidic, myristic and butyric acids), and myristoleic and caproic acids. Hansen et al. (2018) also verified associations between medium-chain fatty acids and the microbiome, however, these associations were for different taxa.

Kato et al. (2017) reported that SFAs were positively correlated with *Fusobacteria* and *Betaproteobacteria* and that vitamin C was also positively correlated to *Fusobacteria.* Our results did not show the same tendency regarding SFAs as they were negatively correlated to *Fusobacteria* and *Betaproteobacteria,* but Vitamin C demonstrated a positive correlation to *Fusobacteria.* However, none of these correlations were statistically significant in our findings.

Studies correlating gut microbiome and dietary habits reported a positive association between *Bacteroidetes and Actinobacteria* with fat and a negative association with fiber, whereas *Firmicutes* and *Proteobacteria* exhibited the opposite correlation (Wu et al., 2011). Although, our results showed that *Bacteroidetes* was positively associated with fat and also with fiber, whereas *Actinobacteria* was positively associated with fiber and negatively to fat. *Firmicutes* and *Proteobacteria* were both negatively associated with fiber and fat. However, none of these findings were statistically significant.

To the best of our knowledge, this study was a pioneer in the assessment of the oral microbiome diversity for a Portuguese sample of type 2 diabetes patients. However, more studies are expected to be made on the oral microbiome category, as this cavity is of easier access when compared to the other regions of the digestive tract. More interesting, however, comes from the evidence that the oral cavity, as the major external entrance of the digestive tract, can harbor a microbiome that most probably will have a great influence on the microbiome ecosystems across the gut. Even though our approach allowed some taxonomic resolution, further investigation regarding type 2 diabetes and diet association with oral microbiome are needed for a better understanding of these associations. The oral microbiome research has an enormous potential to help

to predict some biotic-derived conditions, that ultimately take effect on human health improvement.

6. Conclusions

- In this study we have characterized the oral microbiome of 47 individuals belonging to a control group (25) and a diabetes group (22), where it was possible to identify a biome constituted of 12 phyla, 21 classes, 37 orders, 60 families, 86 genera and 51 species.
- The control group presented a higher number of taxa (202) than the diabetes group (183). 65% of all the identified taxa were shared between the two groups.
- Concerning alpha diversity, although some tendencies were found in a few variables, none of the alpha diversity comparisons between groups or between categories of the variables analyzed were significant.
- Beta diversity measures between the two studied groups were also not significant, therefore, we could not confirm that the microbiome from type 2 diabetes patients was different from the control group, although there were some minor taxonomic differences in class, genus and species level in terms of abundance.
- Diet showed to be an influencing factor, where it was possible to infer some associations between dietary components and the oral microbiome.

7. References

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8. Supplementary material

Supplementary index of figures

Supplementary index of tables

Supplementary document 1. Participant Information Sheet

Código do participante:

FOLHA INFORMATIVA SOBRE O ESTUDO

Título do estudo: ASSOCIAÇÃO ENTRE O MICROBIOMA ORAL E A DIABETES DE MELLITUS TIPO 2

A cavidade oral é das cavidades do corpo humano com uma microbiota mais diversa (conjunto de microrganismos como bactérias, vírus, fungos e outros seres unicelulares). Esses microrganismos, em situações normais, vivem em equilíbrio com o hospedeiro. No entanto, através de fatores desestabilizadores, esse ambiente pode ficar comprometido. Quando isto acontece, o risco de desenvolver certos tipos de doenças aumenta, como é o caso da diabetes de Mellitus. A Diabetes de Mellitus é uma doença metabólica caracterizada por um estado hiperglicémico crónico, que pode manifestar-se na forma de diabetes tipo 1 ou 2. Os indivíduos portadores da diabetes tipo 2 produzem insulina em quantidades insuficientes e podem ainda ser resistentes à sua ação.

São vários os estudos que demonstram a relação disfuncional entre o microbioma oral (conjunto de genomas da microbiota) e a doença Diabetes Mellitus tipo 2. A diabetes apresenta-se como um fator de risco para o desenvolvimento de uma microbiota não saudável, levando ao aparecimento de doenças orais, assim como a presença de uma microbiota disruptiva mostra ser um fator de risco para o aparecimento de diabetes tipo 2.

Desta forma, estamos a convidá-lo(a) a participar numa investigação, no âmbito de uma Dissertação de Mestrado em Ciências do Consumo e Nutrição da Faculdade de Ciências e Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, sobre a relação entre o microbioma oral e a doença Diabetes Mellitus tipo 2. O estudo necessita de duas amostras, um grupo de pacientes com Diabetes Tipo 2 e um grupo controlo (dadores de sangue) para comparação.

Antes de tomar a decisão de se deseja participar ou não, deverá entender o propósito desta investigação e qual será o seu papel nela. Leve o tempo necessário para ler este documento com cuidado. Se não compreender alguma coisa, por favor peça que o/a esclareçam.

Objetivo do Estudo: Compreender a relação entre a doença Diabetes Mellitus tipo 2 e o microbioma oral.

Procedimentos

1. Entrevista

   A entrevista será feita através de um questionário. No questionário irão constar perguntas de carácter demográfico, perguntas de frequência alimentar (com que frequência consome um dado alimento), perguntas sobre restrições alimentares (se não consome um determinado alimento), perguntas referentes ao seu estilo de vida (se fuma ou consome bebidas alcoólicas,…), se está a tomar algum tipo de medicamento e se sim qual, se está a tomar ou esteve sob o efeito de antibióticos e por fim, se sofre de outro tipo de doença(s).

2. Recolha de saliva

   Ser-lhe-á pedido que acumule saliva na boca e que depois a cuspa para um tubo teste. A saliva é o que irá permitir a identificação dos microrganismos presentes na sua cavidade oral. É de elevada importância que não lave os dentes 12 horas antes da recolha. Esta atividade será feita numa única sessão, no Centro Hospitalar de Vila Nova de Gaia/Espinho.

Benefícios

Este tipo de estudo irá permitir aprofundar o conhecimento sobre os microrganismos associados à doença Diabetes Mellitus tipo 2, assim como, possibilitar um melhor conhecimento desta doença. A sua contribuição nesta investigação científica poderá impulsionar avanços científicos que possibilitem, no futuro, uma melhoria da saúde. No entanto, como é um estudo a longo termo, não irá ser beneficiado diretamente.

Confidencialidade dos dados

Toda a informação recolhida no âmbito do estudo será confidencial. O seu nome vai ser representado por um código numérico. A correspondência entre o seu nome e o código vai ficar apenas no laboratório do CIBIO, em local fechado, sem acesso de terceiros. Em todas as análises apenas o código vai ser utilizado. Os resultados das análises e os dados recolhidos poderão ser utilizados em relatórios e/ou publicações, mas serão feitos sempre de forma anónima. Foi pedida autorização à Comissão Nacional de Proteção de Dados (CNPD) para a recolha e uso dos seus dados, garantindo a sua confidencialidade.

Condições e financiamento

O estudo é financiado pelo CIBIO-InBIO (Centro de Investigação em Biodiversidade e Recursos Genéticos). A investigadora principal é a Ana Santos (aluna de mestrado na Faculdade de

Ciências da Universidade do Porto), sob supervisão da Drª Daniela Martins-Mendes do Centro Hospitalar de Vila Nova de Gaia/Espinho. A Ana Santos vai realizar a recolha das amostras e da informação, assim como fazer as análises com a colaboração dos investigadores do CIBIO-InBio Magdalena Gayà, Lucía Perez, Albano Beja Pereira e Jorge Rocha. Em caso de dúvidas por favor não hesite em perguntar qualquer um dos contactos mencionados abaixo.

Contactos

Investigadora principal – Ana Santos
Nº de telemóvel: 919776737
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O estudo foi aprovado pela Comissão de Ética para a Saúde do seu hospital (Centro Hospitalar de Vila Nova de Gaia/Espinho). As comissões de ética são responsáveis pela proteção dos direitos, da segurança e do bem-estar dos participantes nos estudos clínicos.

Indeminização

O presente estudo não apresenta riscos para a sua saúde. Os investigadores assumem as responsabilidades jurídicas e éticas dos procedimentos realizados.

Caso decida não participar

A sua participação neste estudo é completamente voluntária. Cabe-lhe a si decidir se quer ou não participar. Pode recusar-se a participar neste estudo e tal não afetará o seu tratamento e os cuidados médicos a que tem direito, nem a sua relação com o seu médico. Caso decida terminar a sua participação, por favor informe um dos membros do estudo que o acompanhar ao longo desta investigação.

Supplementary document 2. Informed consent form

Código do participante:

FORMULÁRIO DE CONSENTIMENTO INFORMADO

Título do estudo: Associação entre o microbioma oral e a diabetes de mellitus tipo 2

Investigação entre o Centro Hospitalar de Vila Nova de Gaia/Espinho e o CIBIO-InBIO - Centro de Investigação em Biodiversidade e Recursos Genéticos no âmbito de uma Dissertação de Mestrado em Ciências do Consumo e Nutrição da Faculdade de Ciências e Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto. A investigadora principal deste estudo é a Ana Santos (Faculdade de Ciências da Universidade do Porto), sob supervisão da Drª Daniela Martins-Mendes (Centro Hospitalar de Vila Nova de Gaia/Espinho; Faculdade de Medicina da Universidade do Porto; i3S Instituto de Investigação e Inovação em Saúde da Universidade do Porto). Outras pessoas envolvidas neste estudo são: Magdalena Gayà, Lucía Perez, Albano Pereira, e Jorge Rocha do CIBIO.

1. Declaro ter lido e compreendido a folha informativa sobre o estudo, bem como as informações verbais que me foram fornecidas pelos investigadores acima referidos.

2. Compreendo que me foi garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências.

3. Compreendo que tenho o direito de colocar, agora e no desenvolvimento do estudo, qualquer questão sobre o estudo e os métodos a utilizar.

4. Autorizo ser contactado posteriormente pelas investigadoras.

   Sim ☐ Não ☐

5. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelas investigadoras.

Nome do participante: _____

Nº do processo: _____

Assinatura:_____

Nome da Investigadora: … … … … … … … ..... … … … ..... … … … … … … … … … … … …

Assinatura: … … … … … … ..... … … … … … … … … … … … … … **Data:** ..... /..... /.....

Uma cópia deste consentimento é para o participante e outra para o aquivo da investigação.

Caso decida participar no estudo, pedimos-lhe que assine a folha de consentimento anexa.

Supplementary document 3. Questionnaire

Nome da(o) inquirido(a): _____

Data de realização: _____ / _____ / _____ *(dia/mês/ano)*

Local de realização:_____

CÓDIGO DO PARTICIPANTE_____

PROCESSO HOSPITALAR_____

C ____ P_____

TEXTO DE INTRODUÇÃO

Desde já agradecemos a sua cooperação e disponibilidade em participar neste trabalho. Este Questionário é aplicado no âmbito de uma Dissertação de Mestrado em Ciências do Consumo e Nutrição da Faculdade de Ciências e Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, e tem como objetivo avaliar relação entre o microbioma oral e a doença Diabetes Mellitus tipo 2.

Gostaria de lhe colocar algumas perguntas. Este questionário será utilizado unicamente para fins de investigação e as suas respostas serão confidenciais. Aceita dar-nos cerca de 10 a 15 minutos do seu tempo para responder a estas perguntas? Muito obrigada(o).

PARTE A

1. Idade: _____

2. Sexo: Feminino _____ Masculino ____

3. Altura: _____

4. Peso: _____

5. IMC _____

PARTE B - ESTILO DE VIDA

1. Fuma?

Não ☐ Sim ☐ Com que frequência?

-Todos os dias ☐ Quanto?

1 cigarro ☐

Mais de 1 cigarro ☐

1 maço ☐

Mais de um maço ☐

-Quase todos os dias ☐

-Ocasionalmente ☐

2. Consome bebidas alcoólicas?

Não ☐ Sim ☐ Com que frequência?

Todos os dias ☐

Quase todos os dias ☐

Ocasionalmente ☐

3. Possui algum tipo de doença? (no caso dos diabéticos, se possui outra(s) doença(s))

Não ☐ Sim ☐

Qual/Quais?_____
_____
_____
_____

4. Toma algum tipo de medicamento(s)?

Não ☐ Sim ☐ Quais?

Para a diabetes:

_____
_____
_____

Para a hipertensão arterial:

_____
_____
_____

Para a dislipidemia (sangue gordo):

_____
_____
_____

Anti-agregante (para pôr o sangue fino):

_____
_____
_____

Outros:

_____
_____
_____
_____
_____

5. Está a tomar antibióticos? Ou tomou há pouco tempo?

Não ☐ Sim ☐ Há quanto tempo?_____

6. Com que frequência lava os dentes?

Nunca ☐
Depois das refeições ☐
Quando acordo ☐
Quando me vou deitar ☐
Outra ☐ Qual?_____

6.1 Quando foi a última vez que lavou os dentes?_____

7. Pratica exercício físico?

Não ☐ Sim ☐ Com que frequência?

1 vez por semana ☐
2 a 3 vezes por semana ☐
Mais que 3 vezes por semana ☐

PARTE C- HÁBITOS ALIMENTARES

1. Possui algum tipo de restrição alimentar?

Não ☐ Sim ☐ **Qual?_____**

2. Qual foi a sua última refeição?_____

Supplementary document 4. Food Frequency Questionnaire

18535

ID

O questionário seguinte tem como objectivo avaliar a sua alimentação. Por favor, procure responder às questões de uma forma sincera, indicando aquilo que realmente come e não o que gostaria de comer, ou pensa que seria correcto comer.

O questionário pretende identificar o consumo de alimentos do ano anterior. Assim para cada alimento, deve assinalar, no respectivio círculo, quantas vezes por dia, semana ou mês comeu em média , **nos últimos 12 meses**, cada um dos alimentos referidos nesta lista. Não se esqueça de assinalar os alimentos que **nunca** comeu, ou que come **menos de 1 vez por mês** na coluna nunca ou menos de 1 por mês.

Não se esqueça de ter em conta não só as vezes que o alimento é consumido sozinho mas também, aquelas em que é adicionado a outros alimentos ou pratos (ex: o café do café com leite, os ovos das omeletas, etc).

Para os alimentos que só comeu em determinadas épocas do ano (por ex: cerejas ou diospíros), assinale as vezes em que comeu o alimento nessa época, colocando uma cruz **(x)** na **última coluna** (**Sazonal**).

No item nº 86, anote a frequência com que comeu sopa de legumes. Quando consome caldo verde, canja ou sopa instantânea, com uma frequência de **pelo menos 1 vez por semana**, deve assinalar a frequência com que comeu este alimento no quadro existente para "**OUTROS ALIMENTOS**", tendo o cuidado de não o contar na frequência que refere para a sopa de legumes.

Se houver algum alimento não mencionado na lista de alimentos e que tenha consumido pelo menos 1 vez por semana, assinale, no quadro que existe para "**OUTROS ALIMENTOS**", a respectiva frequência e indique a quantidade média que costuma comer de cada vez. **Por ex: frutos tropicais, sumos de fruta natural, farinha de pau, canja, alheiras, cevada, rebuçados, etc**.

Por exemplo: Uma pessoa que bebe leite 2 vezes por dia e o leite que bebe é meio gordo, se a maior parte dos gelados que come é no verão e nessa época come um gelado por dia deve assinalar:

I. PRODUTOS LÁCTEOS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
1. Leite gordo	1 chávena = 250 ml	●	○	○	○	○	○	○	○	○	☐
2. Leite meio-gordo	1 chávena = 250 ml	○	○	○	○	○	○	●	○	○	☐
3. Leite magro	1 chávena = 250 ml	●	○	○	○	○	○	○	○	○	☐
7. Gelados	Um ou 2 bolas	○	○	○	○	○	●	○	○	○	☒

Preencha assim:	Não preencha assim:
● ☒	⊘ ○ ☐ ☑

Por exemplo: se come sopa uma vez por dia, mas 1 vez por semana é canja e não sopa de legumes assinale:

VIII. BEBIDAS E MISCELANEAS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
86. Sopa de legumes	1 prato	○	○	○	○	●	○	○	○	○	☐

OUTROS ALIMENTOS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
CANJA	PRATO	○	○	●	○	○	○	○	○	○	☐

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Pense **nos últimos 12 meses** quantas vezes por dia, semana ou mês, em média, comeu cada um dos alimentos referidos. Não se esqueça de assinalar os alimentos que nunca comeu, ou comeu menos de 1 vez por mês na coluna **(Nunca ou menos de 1 por mês)**.

No grupo **I. PRODUTOS LÁCTEOS** - Não se esqueça de considerar o leite que bebe com o café (**exemplo**: meia de leite, galão,...).

I. PRODUTOS LÁCTEOS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
1. Leite gordo	1 chávena = 250 ml	○	○	○	○	○	○	○	○	○	☐
2. Leite meio-gordo	1 chávena = 250 ml	○	○	○	○	○	○	○	○	○	☐
3. Leite magro	1 chávena = 250 ml	○	○	○	○	○	○	○	○	○	☐
4. Iogurte	Um =125g	○	○	○	○	○	○	○	○	○	☐
5. Queijo (de qualquer tipo incluindo queijo fresco e requeijão)	1 fatia = 30g	○	○	○	○	○	○	○	○	○	☐
6. Sobremesas lácteas: pudim flan, pudim de chocolate, etc	Um ou 1 prato de sobremesa	○	○	○	○	○	○	○	○	○	☐
7. Gelados	Um ou 2 bolas	○	○	○	○	○	○	○	○	○	☐

No grupo **II. OVOS, CARNES E PEIXES** - considere também as vezes que come cada um destes alimentos como elementos de outros pratos, por **exemplo**: o frango do arroz de frango, os ovos das omoletas, as salsichas dos cachorros.

II. OVOS, CARNES E PEIXES	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
8. Ovos	Um	○	○	○	○	○	○	○	○	○	☐
9. Frango	2 peças ou 1/4 de frango	○	○	○	○	○	○	○	○	○	☐
10. Peru, Coelho	1 porção ou 2 peças	○	○	○	○	○	○	○	○	○	☐
11. Carne: vaca, porco,cabrito	1 porção = 120g	○	○	○	○	○	○	○	○	○	☐
12. Figado de vaca, porco, frango	1 porção = 120g	○	○	○	○	○	○	○	○	○	☐
13. Língua, Mão de vaca, Tripas, Chispe, Coração, Rim	1 porção =100g	○	○	○	○	○	○	○	○	○	☐
14. Fiambre, Chouriço, Salpicão, Presunto, etc	2 fatias ou 3 rodelas	○	○	○	○	○	○	○	○	○	☐
15. Salsichas	3 médias	○	○	○	○	○	○	○	○	○	☐
16. Toucinho, Bacon	2 fatias	○	○	○	○	○	○	○	○	○	☐
17. Peixe gordo: sardinha, cavala, carapau, salmão, etc	1 porção =125g	○	○	○	○	○	○	○	○	○	☐
18. Peixe magro: pescada, faneca, dourada, etc	1 porção =125g	○	○	○	○	○	○	○	○	○	☐
19. Bacalhau	1 posta média	○	○	○	○	○	○	○	○	○	☐
20. Peixe conserva: atum, sardinhas,etc	1 lata	○	○	○	○	○	○	○	○	○	☐
21. Lulas, Polvo	1 porção = 100g	○	○	○	○	○	○	○	○	○	☐
22. Camarão, Amêijoas, Mexilhão, etc	1 prato de sobremesa	○	○	○	○	○	○	○	○	○	☐

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No grupo **III. ÓLEOS E GORDURAS** - responda apenas ao que é **adicionado** em saladas, no prato, no pão, etc, e **não** considere a utilizada para cozinhar.

III. ÓLEOS E GORDURAS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
23. Azeite	1 colher de sopa	○	○	○	○	○	○	○	○	○	☐
24. Óleos: girassol, milho, soja	1 colher de sopa	○	○	○	○	○	○	○	○	○	☐
25. Margarina	1 colher de chá	○	○	○	○	○	○	○	○	○	☐
26. Manteiga	1 colher de chá	○	○	○	○	○	○	○	○	○	☐

No grupo **IV. PÃO CEREAIS E SIMILARES** - não se esqueça de considerar também o que come fora das refeições, por **exemplo:** as batatas fritas da refeição e as que come fora das refeições.

IV. PÃO, CEREAIS E SIMILARES	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
27. Pão branco ou Tostas	Um ou 2 tostas	○	○	○	○	○	○	○	○	○	☐
28. Pão (ou tostas), integral,centeio, mistura	Um ou 2 tostas	○	○	○	○	○	○	○	○	○	☐
29. Broa, Broa de avintes	1 fatia = 80g	○	○	○	○	○	○	○	○	○	☐
30. Flocos cereais: muesli, corn-flakes, chocapic,etc.	1 chávena (sem leite)	○	○	○	○	○	○	○	○	○	☐
31. Arroz	½ prato	○	○	○	○	○	○	○	○	○	☐
32. Massas: esparguete, macarrão, etc.	½ prato	○	○	○	○	○	○	○	○	○	☐
33. Batatas fritas caseiras	½ prato	○	○	○	○	○	○	○	○	○	☐
34. Batatas fritas de pacote	1 pacote pequeno	○	○	○	○	○	○	○	○	○	☐
35. Batatas cozidas, assadas, estufadas e puré	2 batatas médias	○	○	○	○	○	○	○	○	○	☐

No grupo **V. DOCES E PASTEIS** - no item 42 (açúcar) considere quantas colheres ou pacotes de açúcar adiciona ao seus alimentos.

V.DOCES E PASTÉIS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
36. Bolachas tipo maria, água e sal ou integrais	3 bolachas	○	○	○	○	○	○	○	○	○	☐
37. Outras bolachas ou Biscoitos	3 bolachas	○	○	○	○	○	○	○	○	○	☐
38. Croissant, Pasteis, Bolicao, Doughnut ou Bolos caseiros	Um; 1 fatia	○	○	○	○	○	○	○	○	○	☐
39.Chocolate (tablete ou em pó)	3 quadrado; 1 colher sopa	○	○	○	○	○	○	○	○	○	☐
40. Snacks de chocolate (Mars, Twix, Kit Kat, etc)	Um	○	○	○	○	○	○	○	○	○	☐
41. Marmelada, Compota, Geleia, Mel	1 colher sobremesa	○	○	○	○	○	○	○	○	○	☐
42. Açúcar	1 colher sobremesa; 1 pacote	○	○	○	○	○	○	○	○	○	☐

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No grupo **VI - HORTALIÇAS E LEGUMES** - responda pensando nos que são **consumidos no prato** (cozidos ou em saladas) e **não** nos que entram na confecção da sopa. Nos que come só numa determinada época do ano não se esqueça de assinalar na coluna sazonal (x).

VI. HORTALIÇAS E LEGUMES	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
43. Couve branca, Couve lombarda	½ chávena	○	○	○	○	○	○	○	○	○	☐
44. Penca, Tronchuda	½ chávena	○	○	○	○	○	○	○	○	○	☐
45. Couve galega	½ chávena	○	○	○	○	○	○	○	○	○	☐
46. Brócolos	½ chávena	○	○	○	○	○	○	○	○	○	☐
47. Couve-flor, Couve-bruxelas	½ chávena	○	○	○	○	○	○	○	○	○	☐
48. Grelos, Nabiças, Espinafres	½ chávena	○	○	○	○	○	○	○	○	○	☐
49. Feijão verde	½ chávena	○	○	○	○	○	○	○	○	○	☐
50. Alface, Agrião	½ chávena	○	○	○	○	○	○	○	○	○	☐
51. Cebola	½ média	○	○	○	○	○	○	○	○	○	☐
52. Cenoura	1 média	○	○	○	○	○	○	○	○	○	☐
53. Nabo	1 médio	○	○	○	○	○	○	○	○	○	☐
54. Tomate fresco	3 rodelas	○	○	○	○	○	○	○	○	○	☐
55. Pimento	6 rodelas	○	○	○	○	○	○	○	○	○	☐
56. Pepino	¼ médio	○	○	○	○	○	○	○	○	○	☐
57. Leguminosas: feijão, grão de bico	1 chávena ou ½ prato	○	○	○	○	○	○	○	○	○	☐
58. Ervilha em grão, Fava	½ chávena ou ¼ prato	○	○	○	○	○	○	○	○	○	☐

No grupo **VII - FRUTOS** - recorde que para os alimentos que **só comeu em determinadas épocas do ano** (por exemplo, cerejas), deve assinalar as vezes em que comeu o alimento nessa época, colocando uma cruz (x) na última coluna (**Sazonal**).

VII. FRUTOS	Porção Média	Frequência alimentar									sazonal
		Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	
59. Maça, pêra	1 média	○	○	○	○	○	○	○	○	○	☐
60. Laranja, Tangerinas	1 média; 2 médias	○	○	○	○	○	○	○	○	○	☐
61. Banana	1 média	○	○	○	○	○	○	○	○	○	☐
62. Kiwi	1 médio	○	○	○	○	○	○	○	○	○	☐
63. Morangos	1 chávena	○	○	○	○	○	○	○	○	○	☐
64. Cerejas	1 chávena	○	○	○	○	○	○	○	○	○	☐
65. Pêssego, Ameixa	1 médio; 3 médias	○	○	○	○	○	○	○	○	○	☐
66. Melão, Melancia	1 fatia média	○	○	○	○	○	○	○	○	○	☐
67. Diospiro	1 médio	○	○	○	○	○	○	○	○	○	☐
68. Figo fresco, Nêsperas,Damascos	3 médios	○	○	○	○	○	○	○	○	○	☐
69. Uvas frescas	1 cacho médio	○	○	○	○	○	○	○	○	○	☐
70. Frutos conserva: pêssego, ananás	2 metades ou rodelas	○	○	○	○	○	○	○	○	○	☐
71. Amêndoas, Avelãs, Nozes, Amendoins, Pistachio, etc.	½ chávena descascado	○	○	○	○	○	○	○	○	○	☐
72. Azeitonas	6 unidades	○	○	○	○	○	○	○	○	○	☐

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No grupo **VIII - BEBIDAS E MISCELANEAS** - neste grupo **não** considere os sumos naturais (estes devem ser registados na tabela "**OUTROS ALIMENTOS**"), não se esqueça dos que são adicionados a outras bebidas, por **exemplo**: considere aqui o café da meia de leite.

VIII. BEBIDAS E MISCELANEAS	Porção Média	Frequência alimentar: Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	sazonal
73. Vinho	1 copo =125ml	○	○	○	○	○	○	○	○	○	□
74. Cerveja	1 garrafa ou 1 lata	○	○	○	○	○	○	○	○	○	□
75. Bebidas brancas: whisky, aguardente, brandy, etc	1 cálice = 40 ml	○	○	○	○	○	○	○	○	○	□
76. Coca-cola, Pepsi-cola ou outras	1 garrafa ou 1 lata	○	○	○	○	○	○	○	○	○	□
77. Ice-tea	1 garrafa ou 1 lata	○	○	○	○	○	○	○	○	○	□
78.Outros refrigerantes, Sumos de fruta ou Néctares embalados	1 garrafa ou 1 copo	○	○	○	○	○	○	○	○	○	□
79.Café (incluindo o adicionado a outras bebidas)	1 chávena café	○	○	○	○	○	○	○	○	○	□
80. Chá preto e verde	1 chávena	○	○	○	○	○	○	○	○	○	□
81. Croquetes, Rissóis, Bolinhos de bacalhau, etc.	3 unidades	○	○	○	○	○	○	○	○	○	□
82. Maionese	1 colher sobremesa	○	○	○	○	○	○	○	○	○	□
83. Molho de tomate, ketchup	1 colher sopa	○	○	○	○	○	○	○	○	○	□
84. Pizza	Meia pizza-média	○	○	○	○	○	○	○	○	○	□
85. Hambúrguer	Um médio	○	○	○	○	○	○	○	○	○	□
86. Sopa de legumes	1 prato	○	○	○	○	○	○	○	○	○	□

Coloque neste quadro informação relativa aos restantes alimentos ou bebidas que não estejam na lista anterior e que tenha consumido pelo menos 1 vez por semana mesmo em pequenas quantidades, ou numa época em particular. Por exemplo: **farinha de pau, canja, alheiras, farinheiras, frutos secos** (figos, ameixas, alperces)**, cevada**, etc.

OUTROS ALIMENTOS	Porção Média	Frequência alimentar: Nunca ou menos de 1 por mês	1 a 3 por mês	1 por semana	2 a 4 por semana	5 a 6 por semana	1 por dia	2 a 3 por dia	4 a 5 por dia	6 ou mais por dia	sazonal
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□
		○	○	○	○	○	○	○	○	○	□

Supplementary table 1. Descriptive statistics of control group's diet**.**

	Control group				
	Mean	**Std. Deviation**	**Median**	**Minimum**	**Maximum**
Protein	94.52	32.17	89.44	33.73	196.78
Carbohydrates	218.47	70.93	232.01	78.94	361.64
Sugar	92.54	31.46	97.81	30.12	141.04
Total fat	73.41	27.23	71.89	27.77	154.13
Saturated fat	20.38	8.68	18.47	7.92	43.53
Monounsaturated fat	34.38	13.26	32.76	11.24	73.09
Polyunsaturated fat	12.41	4.74	11.90	3.93	25.88
Calories	1946.46	584.15	1996.15	818.85	3570.95

Supplementary table 2. Descriptive statistics of diabetes patient's diet.

	Diabetes patients				
	Mean	**Std. Deviation**	**Median**	**Minimum**	**Maximum**
Protein	111.09	35.45	111.17	59.87	211.27
Carbohydrates	236.19	74.98	220.16	138.82	439.53
Sugar	84.02	32.6	78.93	33.68	168.17
Total fat	85.02	35.79	72.06	43.84	170.42
Saturated fat	21.87	8.04	19.57	13.00	46.08
Monounsaturated fat	40.36	18.99	32.49	17.79	83.39
Polyunsaturated fat	15.26	7.49	11.80	7.04	33.25
Calories	2199.83	726.42	1960.28	1333.11	4037.56

Supplementary table 3. Descriptive statistics of control group's diet according to sex.

		Control group				
		Mean	**Std. Deviation**	**Median**	**Minimum**	**Maximum**
Protein	**Male**	85.03	20.34	89.41	33.73	113.38
	Female	114.69	43.79	109.71	75.31	196.78
Carbohydrates	**Male**	210.57	69.00	230.91	78.94	296.86
	Female	235.27	76.75	225.82	92.82	361.64
Sugar	**Male**	87.14	29.73	97.76	30.12	121.11
	Female	104.00	33.94	116.41	49.49	141.04
Total fat	**Male**	65.87	21.13	66.17	27.77	104.81
	Female	89.42	33.02	82.14	46.11	154.13
Saturated fat	**Male**	18.35	7.17	16.2	7.92	34.76
	Female	24.68	10.46	22.38	10.94	43.53
Monounsaturated fat	**Male**	30.97	10.97	31.19	11.24	51.01
	Female	41.62	15.48	37.40	23.26	73.09
Polyunsaturated fat	**Male**	10.85	3.37	11.47	3.93	17.26
	Female	15.73	5.72	15.04	7.77	25.88
Calories	**Male**	1832.65	483.26	1905.18	818.85	2443.47
	Female	2188.32	733.37	2110.65	1078.7	3570.95

Supplementary table 4. Descriptive statistics of diabetes group's diet according to sex.

		Diabetes patients				
		Mean	**Std. Deviation**	**Median**	**Minimum**	**Maximum**
Protein	**Male**	113.16	34.06	111.70	59.87	211.27
	Female	106.68	40.30	84.84	61.69	166.16
Carbohydrates	**Male**	232.95	69.48	220.16	138.82	406.89
	Female	243.04	90.32	217.45	166.43	439.53
Sugar	**Male**	80.90	31.50	78.93	33.68	150.98
	Female	90.67	36.10	80.29	61.04	168.17

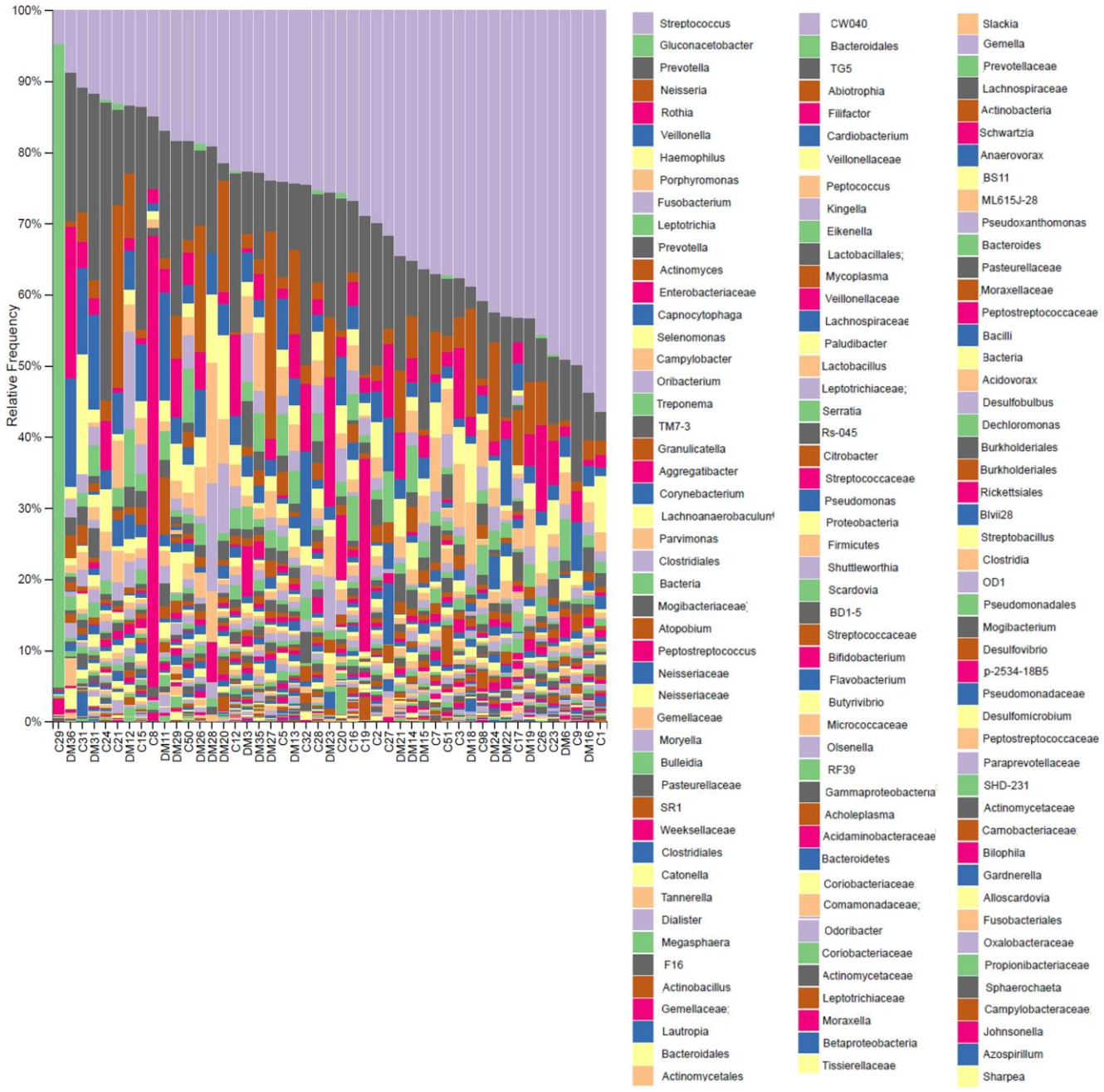
Total fat	**Male**	80.02	27.14	72.06	42.84	156.55
	Female	95.62	50.18	72.72	47.09	170.42
Saturated fat	**Male**	21.08	6.23	19.57	15.14	40.72
	Female	23.53	11.31	19.45	13.00	46.08
Monounsaturated fat	**Male**	37.42	14.15	32.49	17.79	68.76
	Female	46.60	26.71	32.58	20.54	83.39
Polyunsaturated fat	**Male**	14.27	6.16	11.80	7.04	33.25
	Female	17.35	9.90	13.74	7.44	32.23
Calories	**Male**	2173.00	624.87	1992.20	1390.72	4037.56
	Female	2256.82	954.62	1771.49	1333.11	3888.37

Supplementary table 5. Descriptive statistics of control and diabetes groups' BMI.

		Mean	Std. Deviation	Median	Minimum	Maximum
Underweight	**Control**	-	-	-	-	-
	Diabetes	18.20	0.00	18.20	-	-
Normal weight	**Control**	22.55	1.20	22.65	20.10	24.20
	Diabetes	23.74	1.40	24.40	21.20	25.00
Preobese	**Control**	27.03	1.35	26.75	25.30	29.40
	Diabetes	27.31	1.42	27.00	25.50	29.60
Obese class I	**Control**	31.53	1.06	31.55	30.10	32.90
	Diabetes	32.08	1.40	32.20	30.10	33.80
Obese class II	**Control**	-	-	-	-	-
	Diabetes	38.90	0.51	39.10	38.90	39.40
Obese class III	**Control**	41.90	0.00	41.90	-	-
	Diabetes	-	-	-	-	-

Supplementary table 6. Descriptive statistics of control and diabetes groups' BMI according to sex.

			Mean	Std. Deviation	Median	Minimum	Maximum
Underweight	**Diabetes**	**Male**	18.20	0.00	18.20	-	-
		Female	-	-	-	-	-
Normal weight	**Control**	**Male**	22.70	1.21	22.70	20.10	24.20
		Female	21.50	0.00	21.50		
	Diabetes	**Male**	23.74	1.40	24.40	21.20	25.00
		Female	-	-	-	-	-
Preobese	**Control**	**Male**	27.34	1.53	27.35	25.30	29.40
		Female	26.43	0.48	26.65	25.60	26.80
	Diabetes	**Male**	27.38	1.26	27.00	25.70	29.30
		Female	27.10	1.79	26.20	25.50	29.60
Obese class I	**Control**	**Male**	31.55	0.55	31.55	31.00	32.10
		Female	31.50	1.40	31.50	30.10	32.90
	Diabetes	**Male**	31.50	1.40	31.50	31.10	32.90
		Female	32.65	1.15	32.65	31.50	33.80
Obese class II	**Diabetes**	**Male**	-	-	-	-	-
		Female	38.90	0.51	39.10	38.20	39.40
Obese class III	**Control**	**Male**	-	-	-	-	-
		Female	41.90	0.00	41.90	-	-



Supplementary Figure 1. The most abundant taxa up to the genus level per subject. Sample names starting with C are from to the control group and those starting with DM are from the diabetics group

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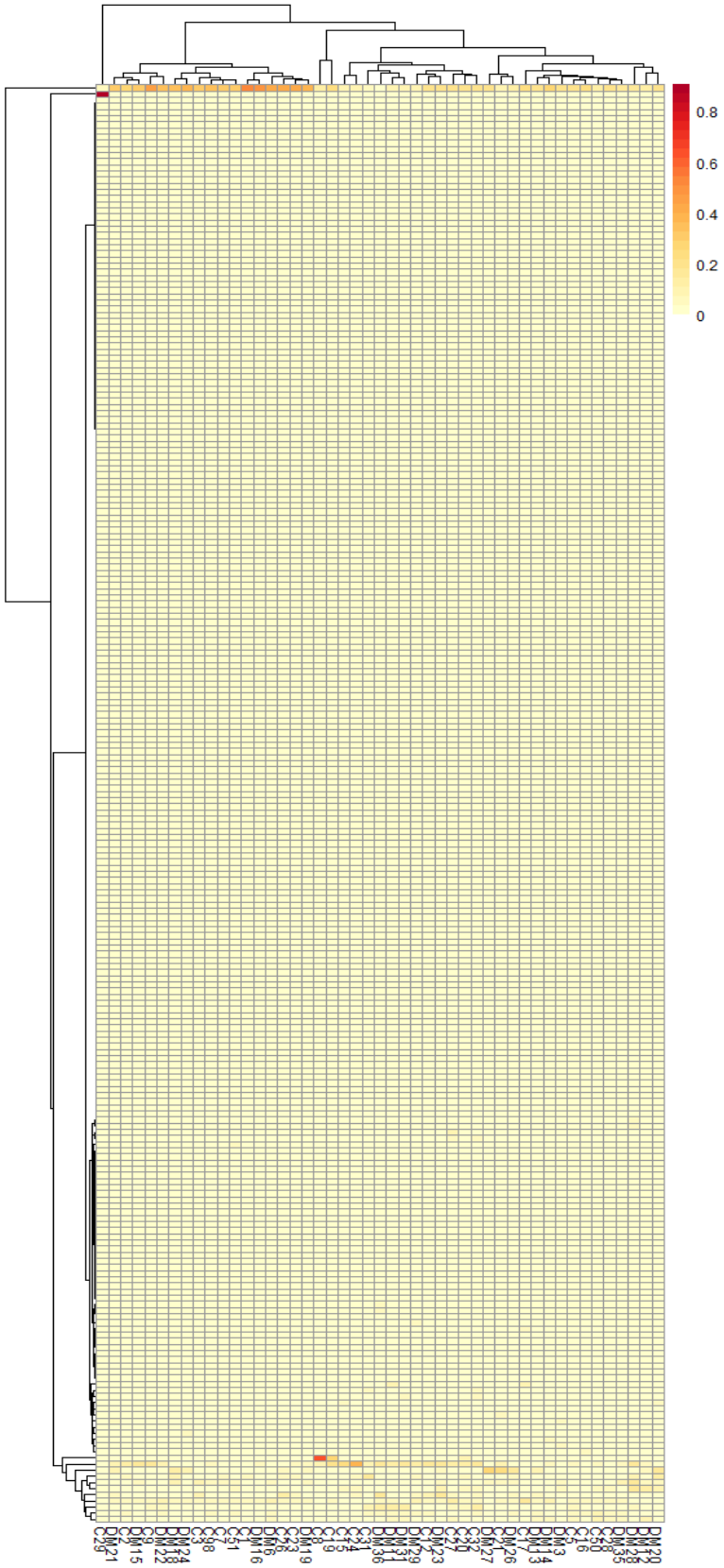

Supplementary Figure 2. Heatmap of the relative abundance of the 233 taxa. Each column of the heatmap represents the individuals from control (C) and diabetes (DM) groups and the rows are the taxa up to the species level. The vertical color bar on the right side of the graph defines the relative abundance of each taxon. Taxa names are present in the supplementary table 7.

Supplementary Table 7. Taxa names corresponding to supplementary figure 2.

order	Taxa	order	Taxa	order	Taxa
1	*Streptococcus*	78	*TG5 spp.*	155	*Acidovorax*
2	*Prevotella melaninogenica*	79	*Kingella spp.*	156	*Streptobacillus spp.*
3	*Streptococcus spp.*	80	*Bacteroidales*	157	*Serratia ureilytica*
4	*Neisseria*	81	*Peptococcus spp.*	158	*Selenomonas*
5	*Porphyromonas spp.*	82	*Treponema amylovorum*	159	*Dechloromonas spp.*
6	*Rothia mucilaginosa*	83	*Eikenella spp.*	160	*Pseudomonadales*
7	*Fusobacterium spp.*	84	*Lactobacillales*	161	*Burkholderiales*
8	*Haemophilus parainfluenzae*	85	*Treponema socranskii*	162	*Lactobacillus mucosae*
9	*Veillonella dispar*	86	*Lachnospiraceae*	163	*ML615J-28*
10	*Prevotella*	87	*Cardiobacterium spp.*	164	*Peptostreptococcaceae*
11	*Leptotrichia*	88	*Serratia marcescens*	165	*Burkholderiales*
12	*Enterobacteriaceae*	89	*Mycoplasma spp.*	166	*Lactobacillus helveticus*
13	*Gluconacetobacter spp.*	90	*Paludibacter spp.*	167	*Clostridia*
14	*Actinomyces;*	91	*Streptococcus anginosus*	168	*Blvii28 spp.*
15	*Prevotella*	92	*Leptotrichiaceae*	169	*Desulfobulbus spp.*
16	*Capnocytophaga*	93	*Rothia spp.*	170	*Olsenella*
17	*Selenomonas*	94	*Citrobacter*	171	*BS11*
18	*Oribacterium*	95	*Scardovia spp.*	172	*OD1*
19	*TM7-3*	96	*Lactobacillus spp.*	173	*Actinomyces*
20	*Veillonella parvula*	97	*Pseudomonas spp.*	174	*Mogibacterium spp.*
21	*Neisseria*	98	*Firmicutes*	175	*Pseudomonadaceae*
22	*Campylobacter*	99	*Micrococcaceae*	176	*Paraprevotellaceae*
23	*Granulicatella*	100	*Streptococcaceae*	177	*Gardnerella spp.*
24	*Parvimonas spp.*	101	*Proteobacteria*	178	*Abiotrophia*
25	*Prevotella pallens*	102	*Actinobacillus spp.*	179	*Desulfomicrobium orale*
26	*Lachnoanaerobaculum orale*	103	*Rs-045*	180	*Actinobacillus delphinicola*
27	*Rothia dentocariosa*	104	*Streptococcaceae*	181	*Bilophila*
28	*Bacteria*	105	*Aggregatibacter*	182	*Rickettsiales*

29	*Clostridiales*	106	*Shuttleworthia satelles*	183	*p-2534-18B5*
30	*Atopobium spp.*	107	*BD1-5*	184	*Actinomyces hyovaginalis*
31	*Peptostreptococcus spp.*	108	*Bulleidia spp.*	185	*Desulfovibrio spp.*
32	*Aggregatibacter segnis*	109	*Atopobium*	186	*Oxalobacteraceae*
33	*Mogibacteriaceae*	110	*Butyrivibrio spp.*	187	*Alloscardovia spp.*
34	*Corynebacterium spp.*	111	*RF39*	188	*Propionibacteriaceae*
35	*Neisseriaceae*	112	*Bifidobacterium spp.*	189	*SHD-231 spp.*
36	*Moryella spp.*	113	*Coriobacteriaceae*	190	*Actinomycetaceae*
37	*Treponema spp.*	114	*Coriobacteriaceae*	191	*Flavobacteriaceae*
38	*Neisseriaceae*	115	*Lactobacillus reuteri*	192	*TM7*
39	*Veillonella*	116	*Flavobacterium succinicans*	193	*Agrobacterium spp.*
40	*Gemellaceae*	117	*Olsenella profusa*	194	*Campylobacteraceae*
41	*Prevotella nanceiensis*	118	*Comamonadaceae*	195	*Azospirillum spp.*
42	*Bulleidia moorei*	119	*Bacteroidetes*	196	*Erysipelotrichaceae*
43	*SR1*	120	*Acidaminobacteraceae*	197	*Neisseria subflava*
44	*Pasteurellaceae*	121	*Slackia spp.*	198	*Peptostreptococcus*
45	*Rothia*	122	*Gemella spp.*	199	*Pyramidobacter piscolens*
46	*Clostridiales*	123	*Peptostreptococcaceae*	200	*Fusobacteriales*
47	*Weeksellaceae*	124	*Lactobacillus*	201	*Sediminibacterium*
48	*Megasphaera spp.*	125	*Bifidobacterium longum*	202	*Burkholderiaceae*
49	*Prevotella tannerae*	126	*Acholeplasma spp.*	203	*Wolinella succinogenes*
50	*Catonella spp.*	127	*Cardiobacterium valvarum*	204	*Sharpea*
51	*F16*	128	*Neisseria bacilliformis*	205	*Abiotrophia defectiva*
52	*Prevotella nigrescens*	129	*Atopobium rimae*	206	*Legionellales*
53	*Dialister spp.*	130	*Gammaproteobacteria*	207	*Serratia*
54	*Tannerella spp.*	131	*Betaproteobacteria*	208	*Bacillales*
55	*Actinomycetales*	132	*Leptotrichiaceae*	209	*Polyangium brachysporum*
56	*Haemophilus*	133	*Flavobacterium spp.*	210	*Actinomycetales*
57	*Gemellaceae*	134	*Actinomycetaceae*	211	*Porphyromonadaceae*

58	*Lautropia spp.*	135	*Odoribacter spp.*	212	*Pasteurella aerogenes*
59	*Porphyromonas endodontalis*	136	*Cardiobacterium*	213	*Sphaerochaeta spp.*
60	*Prevotella intermedia*	137	*Pseudomonas*	214	*Campylobacterales*
61	*CW040*	138	*Moraxella spp.*	215	*Enterobacteriaceae*
62	*Prevotella*	139	*Tissierellaceae*	216	*Johnsonella ignava*
63	*Campylobacter*	140	*Pasteurellaceae*	217	*Adlercreutzia spp.*
64	*Corynebacterium durum*	141	*Lachnospiraceae*	218	*Syntrophomonas spp.*
65	*Bacteroidales*	142	*Prevotellaceae*	219	*Treponema*
66	*Actinobacillus parahaemolyticus*	143	*Pseudoxanthomonas mexicana*	220	*Bacillales*
67	*Veillonellaceae*	144	*Capnocytophaga*	221	*Erysipelotrichaceae*
68	*Veillonella spp.*	145	*Actinobacteria*	222	*Neisseria cinerea*
69	*Haemophilus spp.*	146	*Olsenella uli*	223	*Porphyromonas*
70	*Rothia aeria*	147	*Bacteria*	224	*Peptococcaceae*
71	*Neisseria oralis*	148	*Lactobacillus zeae*	225	*Massilia*
72	*Capnocytophaga ochracea*	149	*Anaerovorax*	226	*Corynebacterium*
73	*Filifactor spp.*	150	*Campylobacter rectus*	227	*Tissierellaceae*
74	*Abiotrophia spp.*	151	*Bacilli*	228	*Fusobacteriaceae*
75	*Selenomonas noxia*	152	*Bacteroides spp.*	229	*Aerococcaceae*
76	*Veillonellaceae*	153	*Schwartzia spp.*	230	*Pasteurella*
77	*Aggregatibacter spp.*	154	*Moraxellaceae*	231	*Carnobacteriaceae*
		232	*Granulicatella balaenopterae*		
		233	*Bifidobacteriaceae*		

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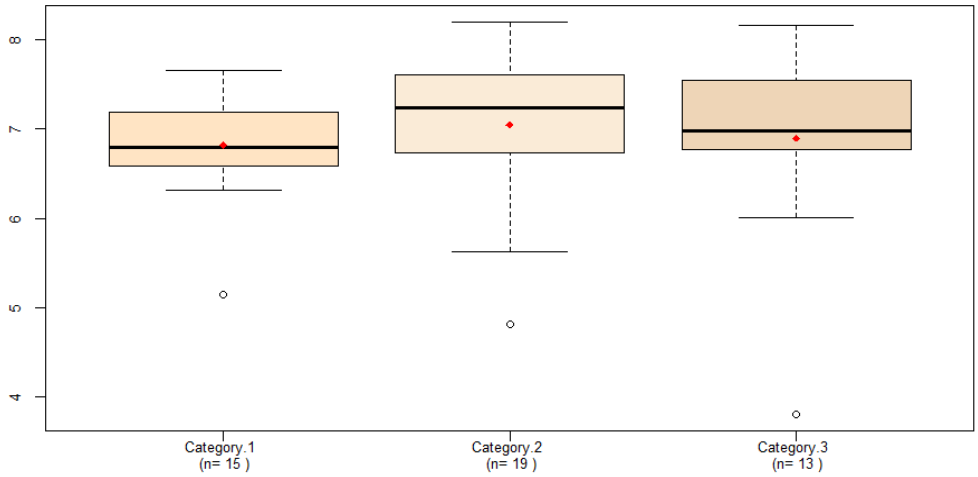
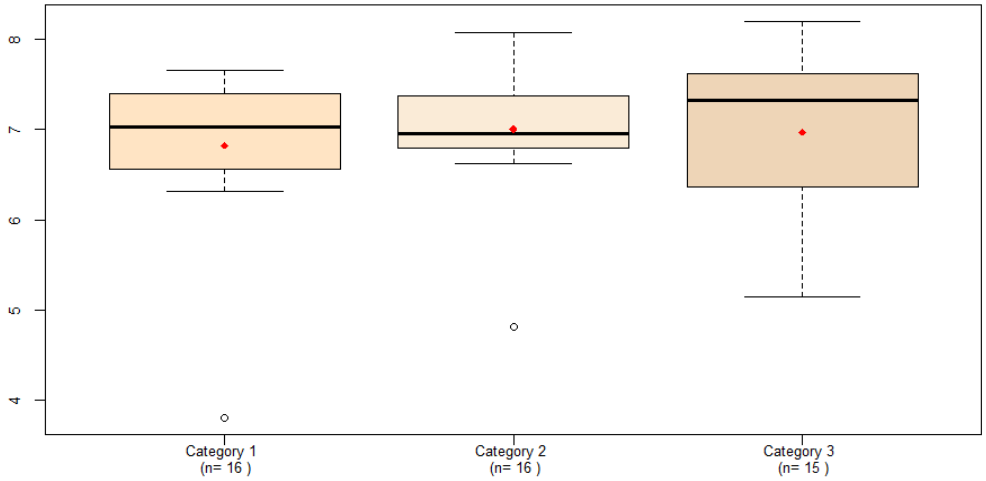
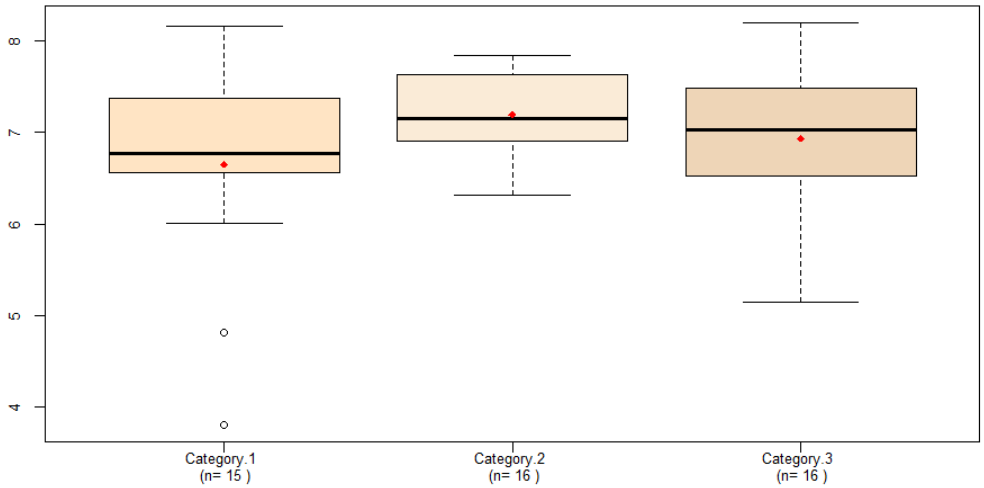


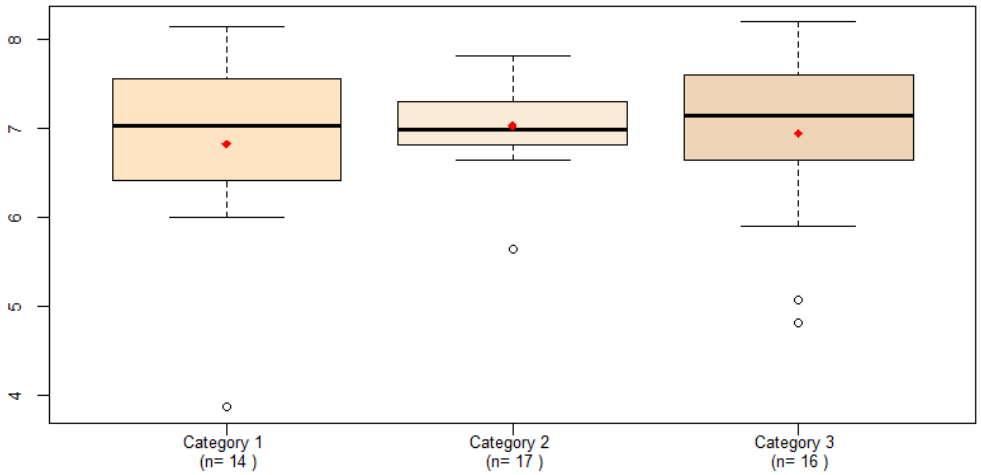
Supplementary Figure 3. Boxplots depicting Shannon index distributions according to age; Red dot represents the mean of each group.



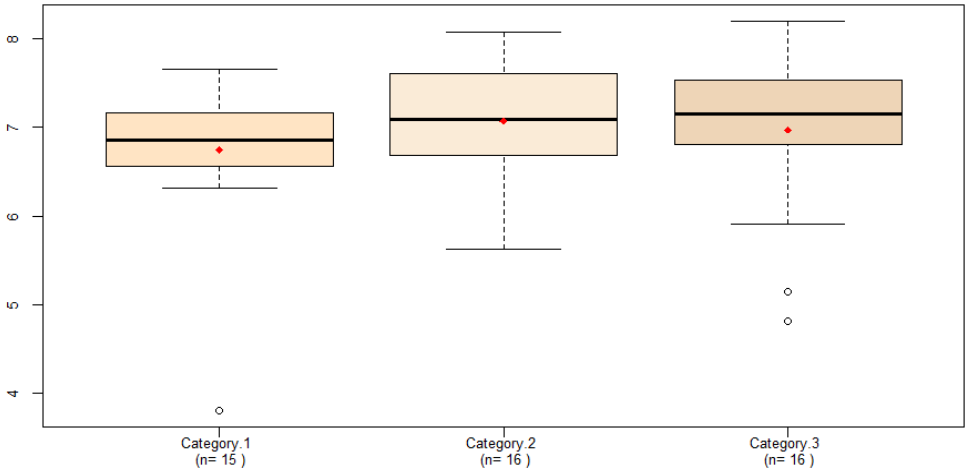
Supplementary Figure 4. Boxplots depicting Shannon index distributions according to carbohydrates consumption; Red dot represents the mean of each group



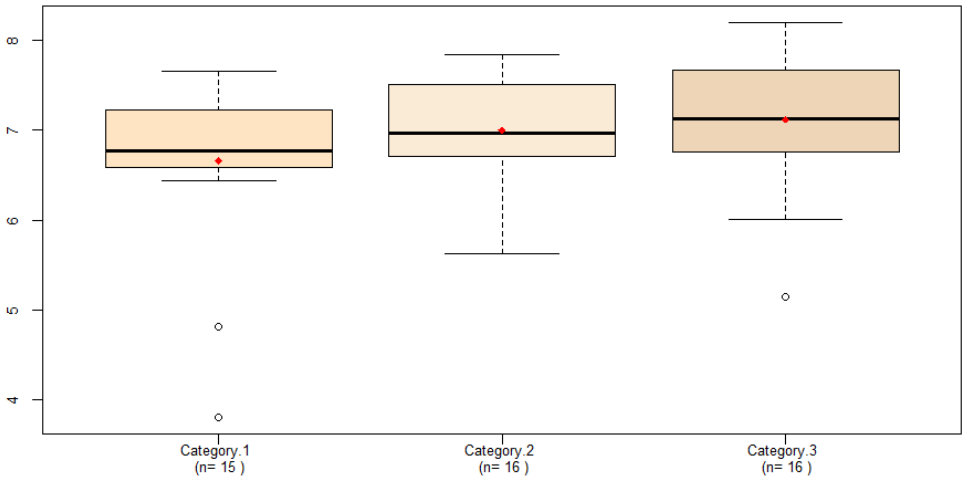
Supplementary Figure 5 Boxplots depicting Shannon index distributions according to coffee consumption; Red dot represents the mean of each group.



Supplementary Figure 6. Boxplots depicting Shannon index distributions according to total fat consumption; Red dot represents the mean of each group.



Supplementary Figure 7. Boxplots depicting Shannon index distributions according to energy intake; Red dot represents the mean of each group.



Supplementary Figure 8. Boxplots depicting Shannon index distributions according to sugar consumption; Red dot represents the mean of each group.

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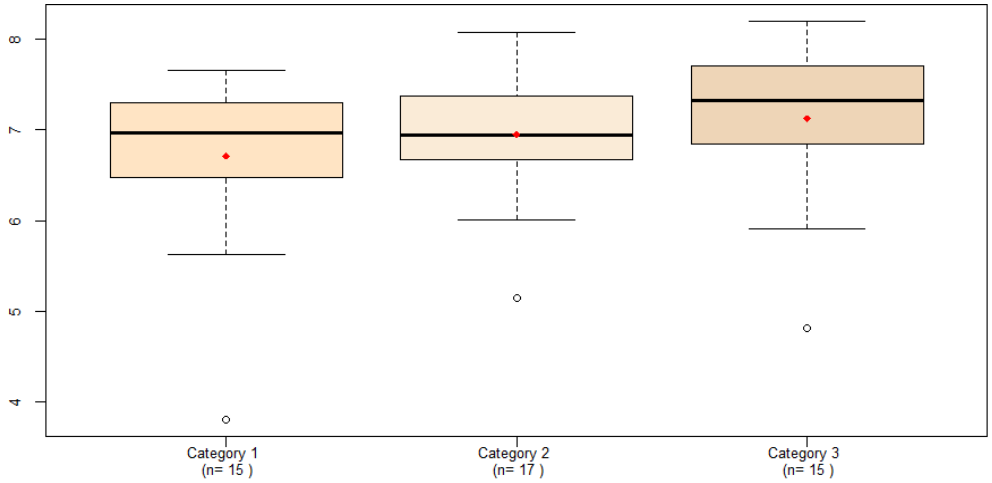
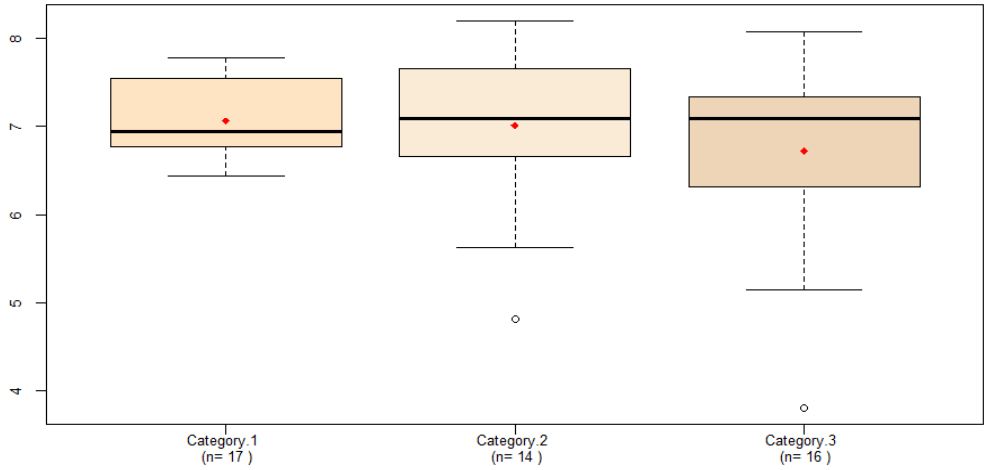
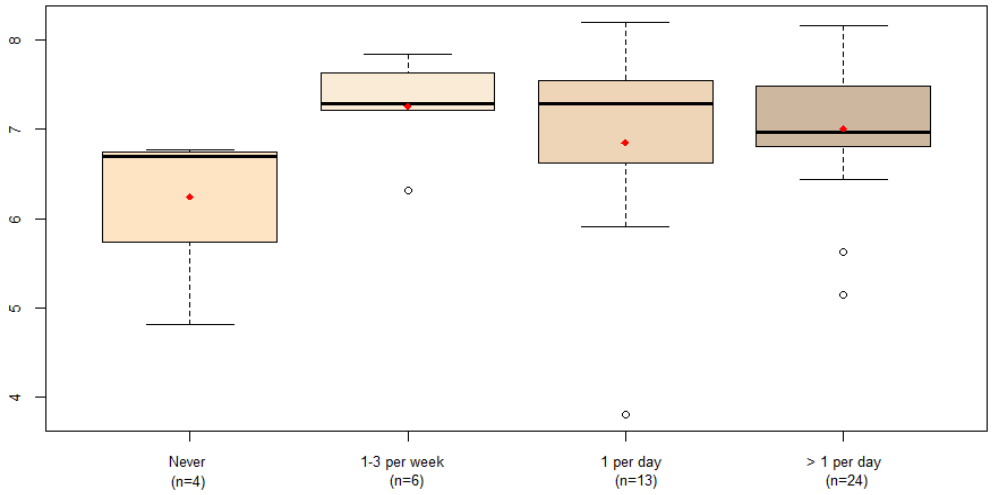


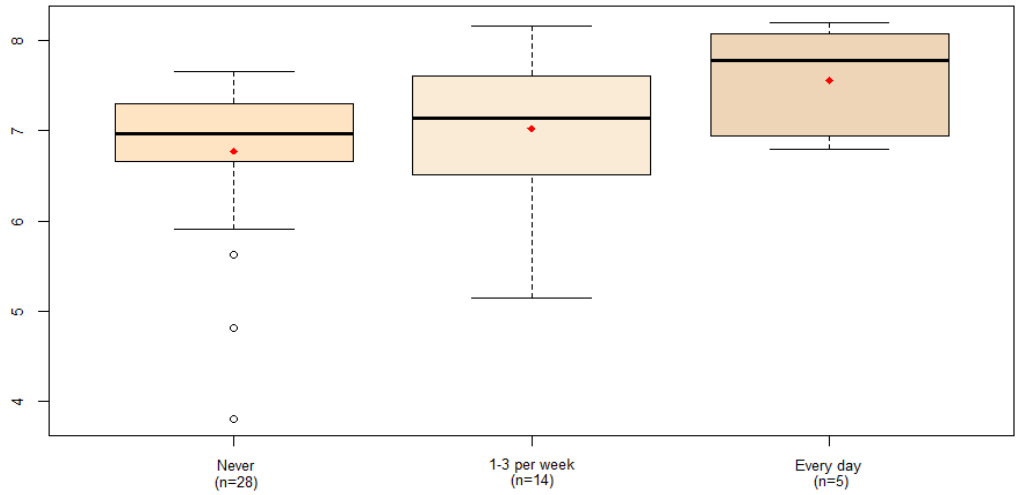
Supplementary Figure 9. Boxplots depicting Shannon index distributions according to protein consumption; Red dot represents the mean of each group



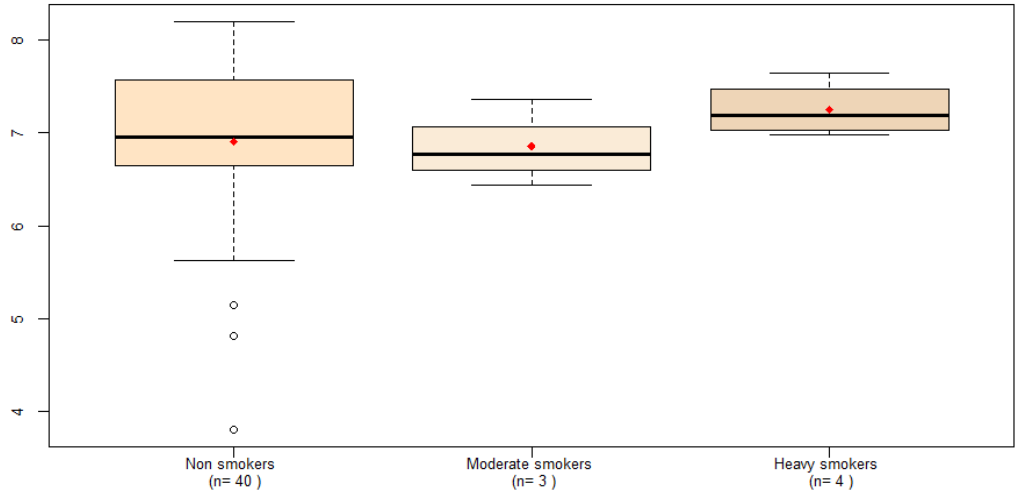
Supplementary Figure 10. Boxplots depicting Shannon index distributions according to alcohol consumption; Red dot represents the mean of each group.



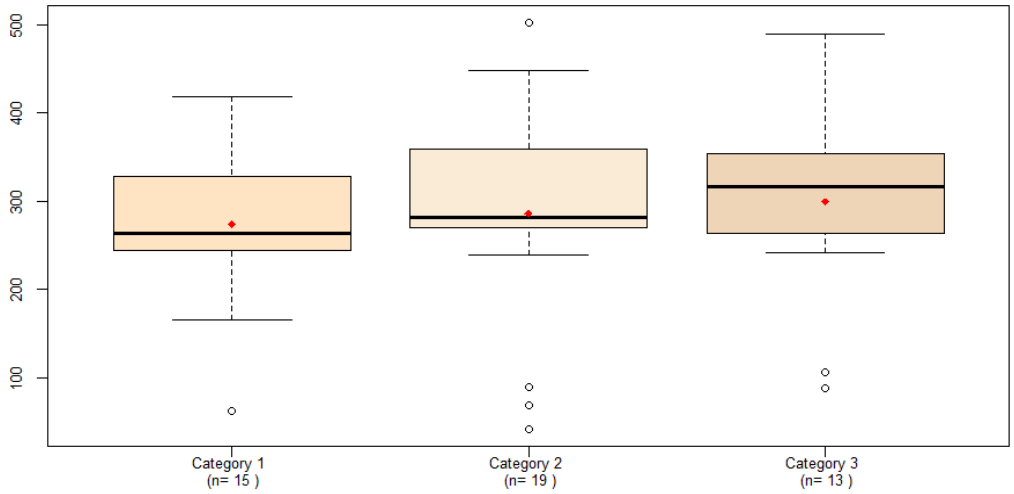
Supplementary Figure 11. Boxplots depicting Shannon index distributions according to teeth brushing habits; Red dot represents the mean of each group.



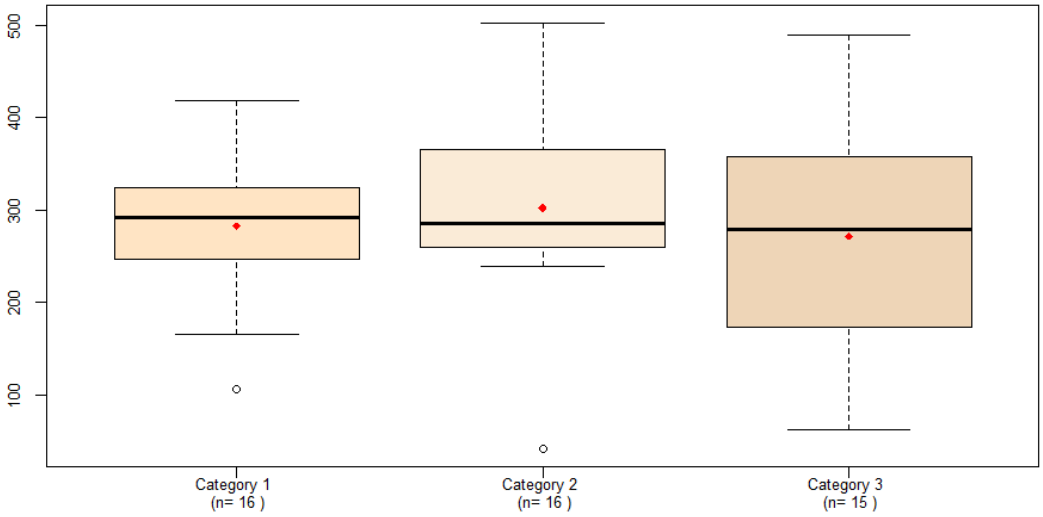
Supplementary Figure 12. Boxplots depicting Shannon index distributions according to mouthwash habits; Red dot represents the mean of each group.



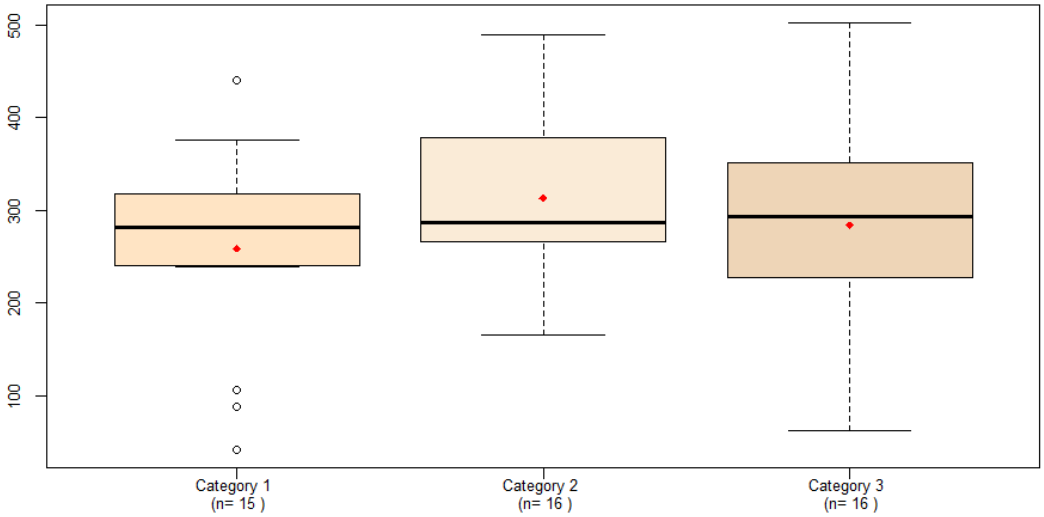
Supplementary Figure 13. Boxplots depicting Shannon index distributions according to smoking habits; Red dot represents the mean of each group.



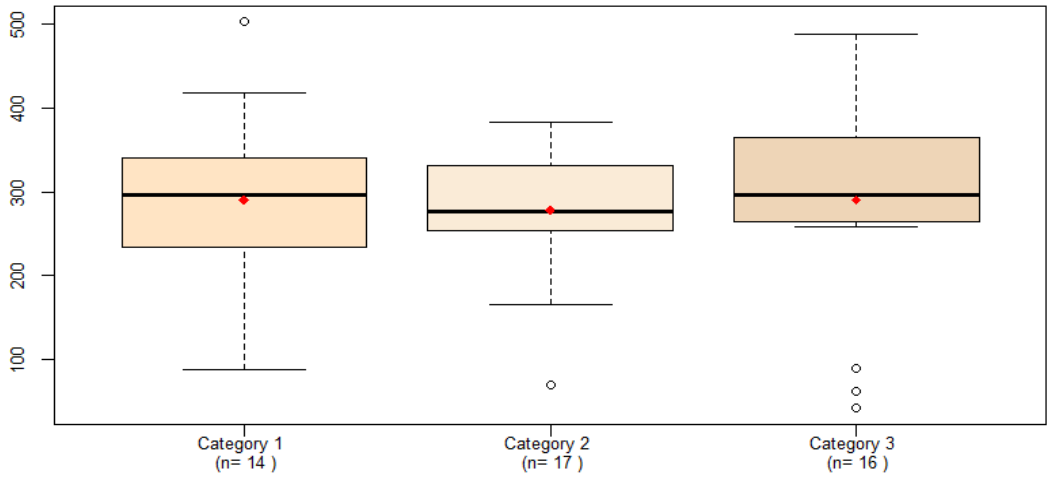
Supplementary Figure 14. Boxplots depicting ASVs abundance distributions according to age; Red dot represents the mean of each group.



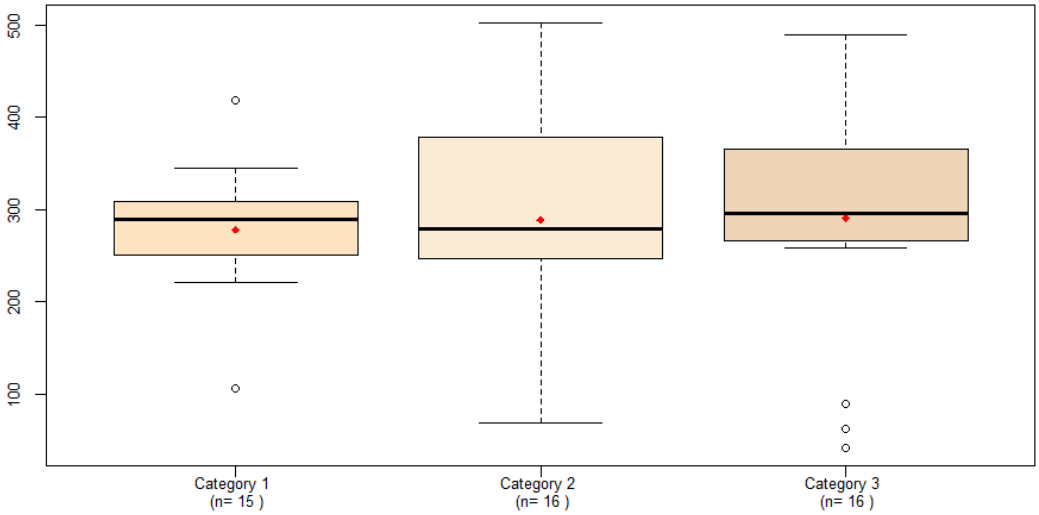
Supplementary Figure 15. Boxplots depicting ASVs abundance distributions according to carbohydrates consumption; Red dot represents the mean of each group.



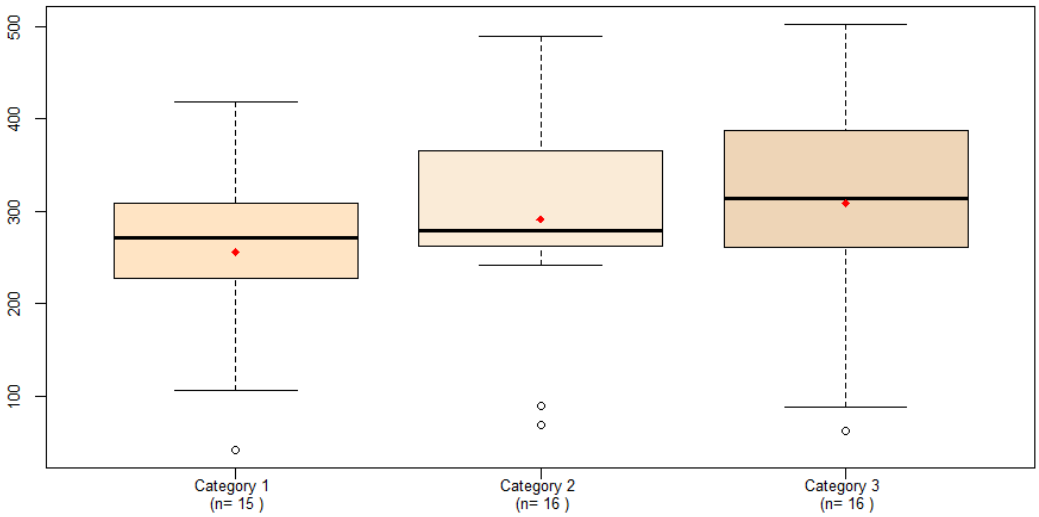
Supplementary Figure 16. Boxplots depicting ASVs abundance distributions according to coffee consumption; Red dot represents the mean of each group.



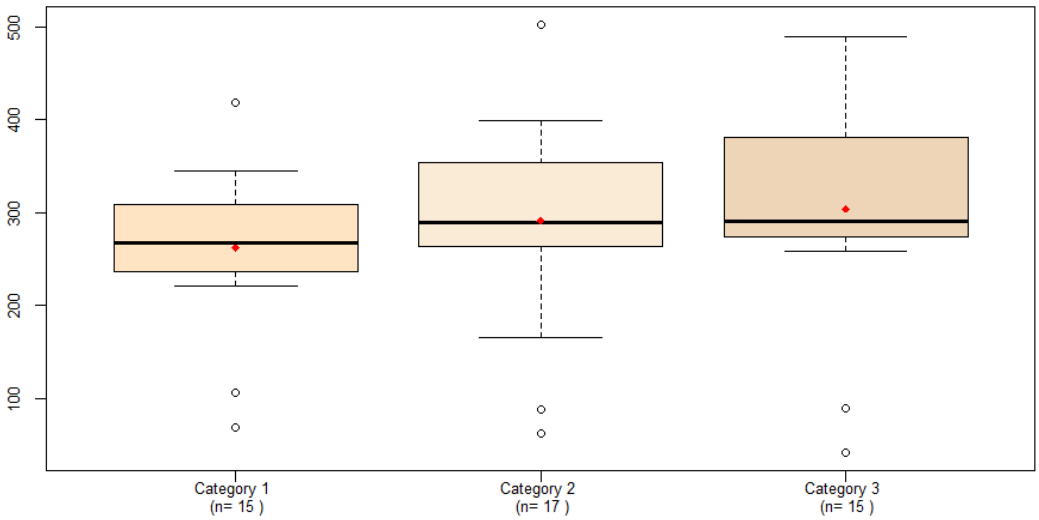
Supplementary Figure 17. Boxplots depicting ASVs abundance distributions according to total fat consumption; Red dot represents the mean of each group.



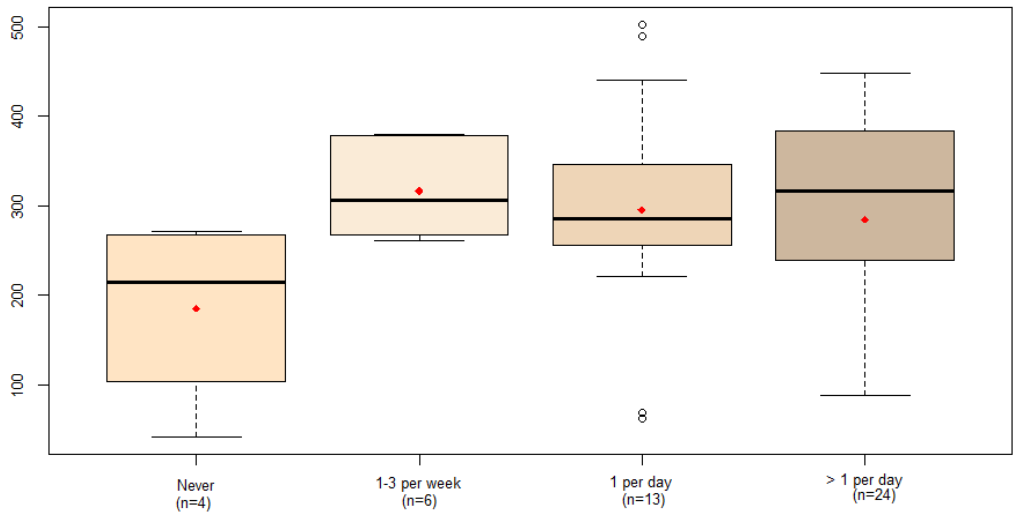
Supplementary Figure 18. Boxplots depicting ASVs abundance distributions according to energy intake; Red dot represents the mean of each group.



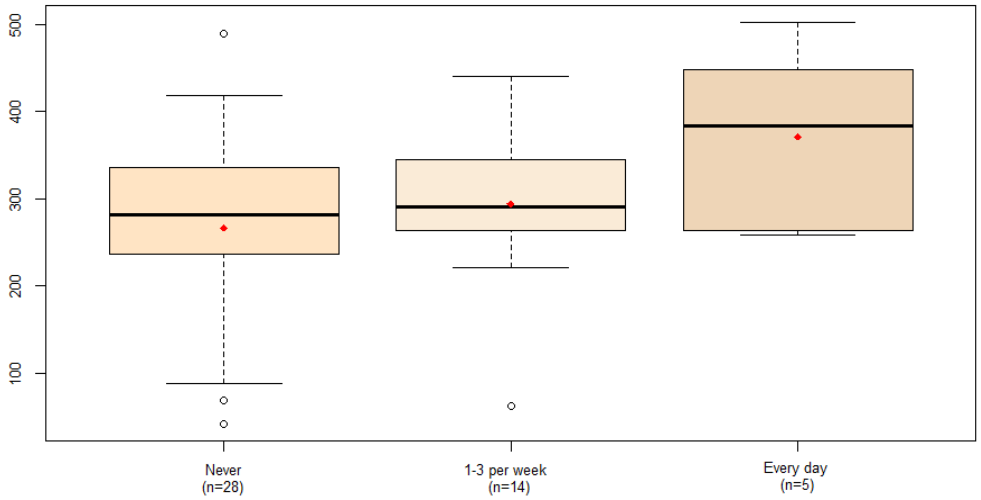
Supplementary Figure 19. Boxplots depicting ASVs abundance distributions according to sugar consumption; Red dot represents the mean of each group.



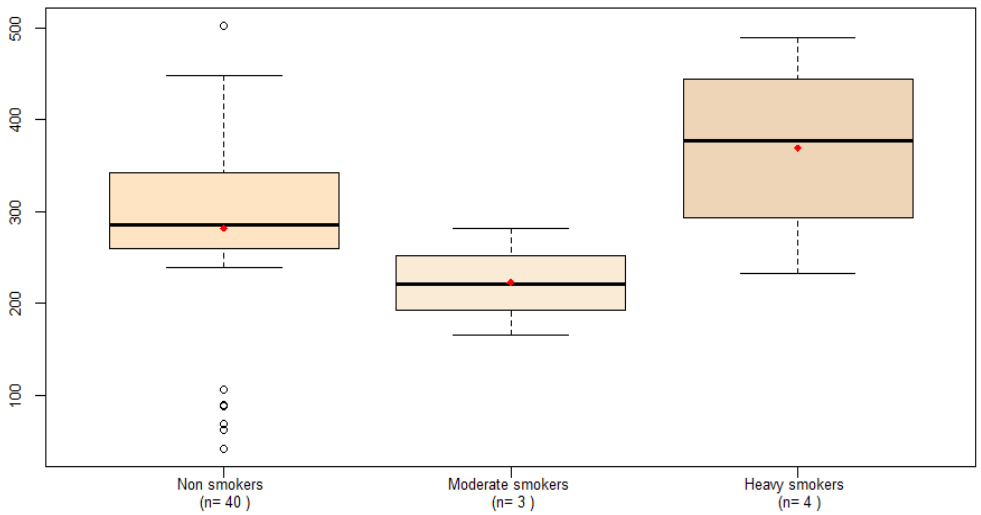
Supplementary Figure 20. Boxplots depicting ASVs abundance distributions according to protein consumption; Red dot represents the mean of each group



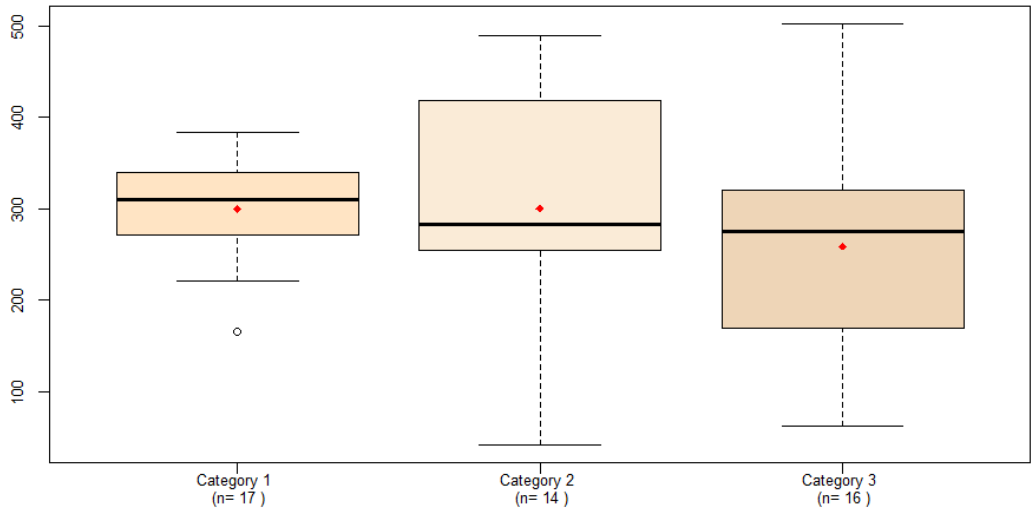
Supplementary Figure 21. Boxplots depicting ASVs abundance distributions according to teeth brushing habits; Red dot represents the mean of each group.



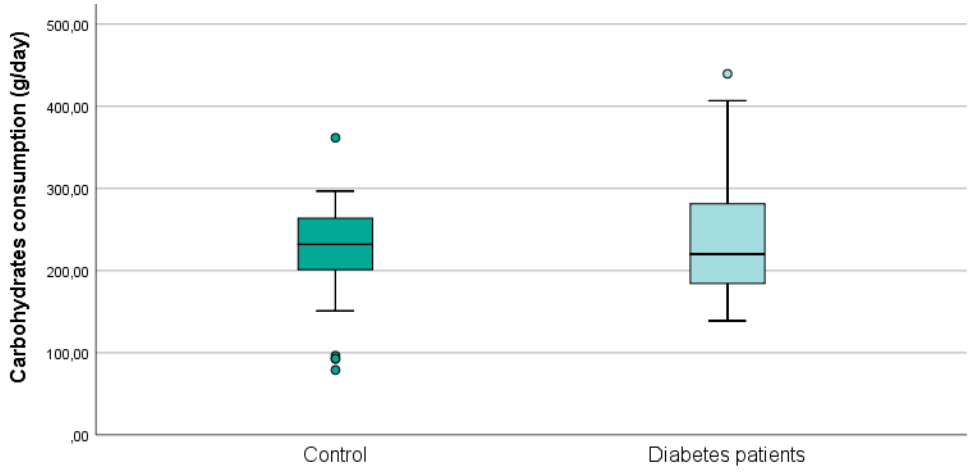
Supplementary Figure 22. Boxplots depicting ASVs abundance distributions according to mouthwash habits; Red dot represents the mean of each group.



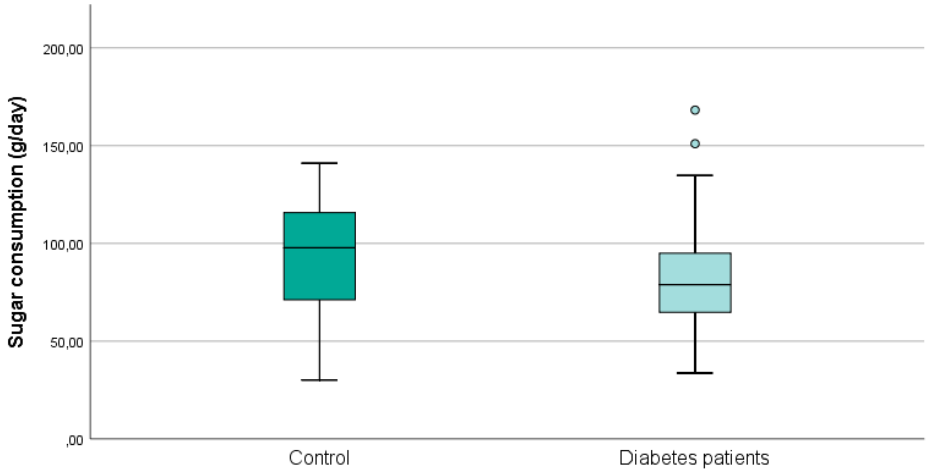
Supplementary Figure 23 Boxplots depicting ASVs abundance distributions according to smoking habits; Red dot represents the mean of each group.



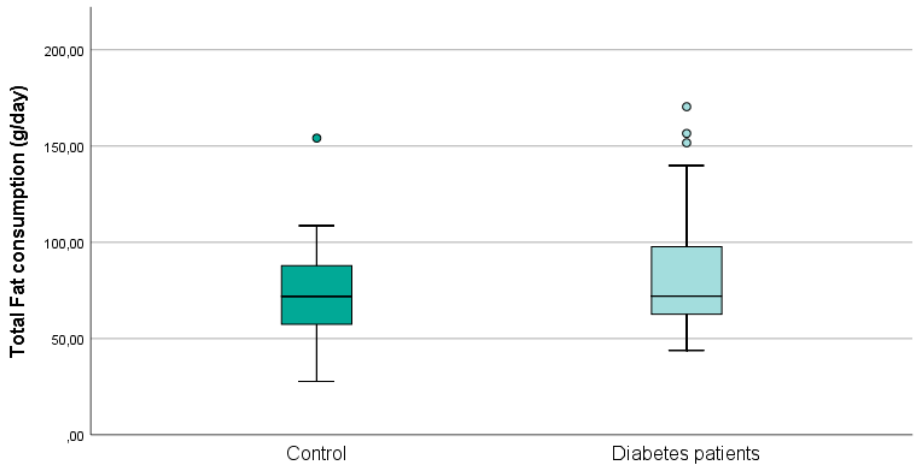
Supplementary Figure 24. Boxplots depicting ASVs abundance distributions according to alcohol consumption; Red dot represents the mean of each group.



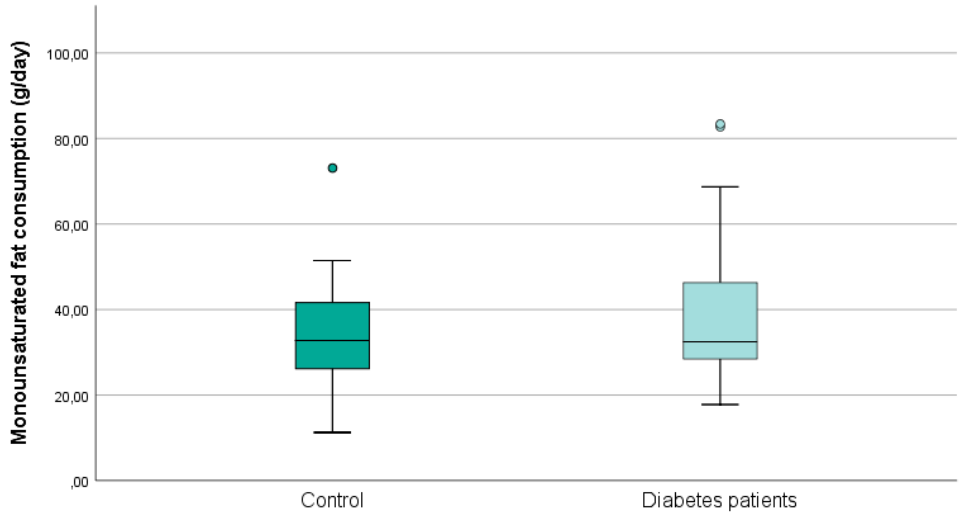
Supplementary Figure 25. Carbohydrates consumption (g/day) in both diabetes patients and control groups.



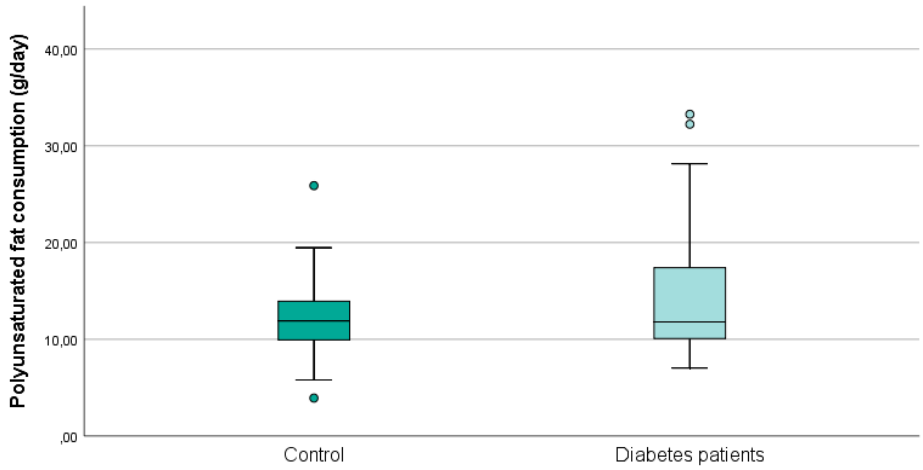
Supplementary Figure 26. Sugar consumption (g/day) in both diabetes patients and control groups.



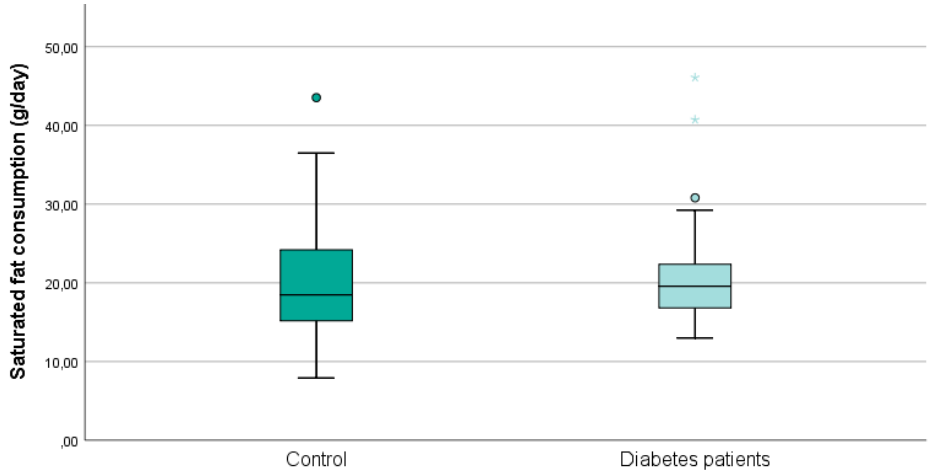
Supplementary Figure 27. Total fat consumption (g/day) in both diabetes patients and control groups.



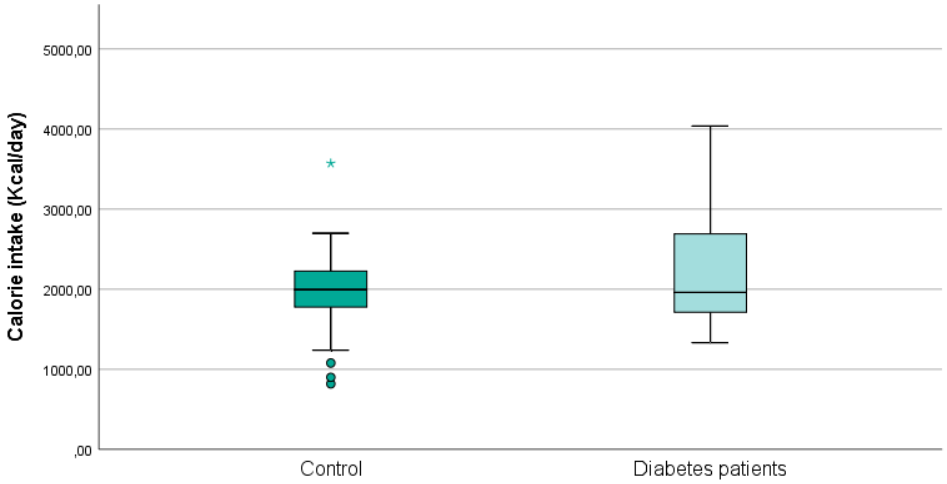
Supplementary Figure 28. Monounsaturated fat consumption (g/day) in both diabetes patients and control groups.



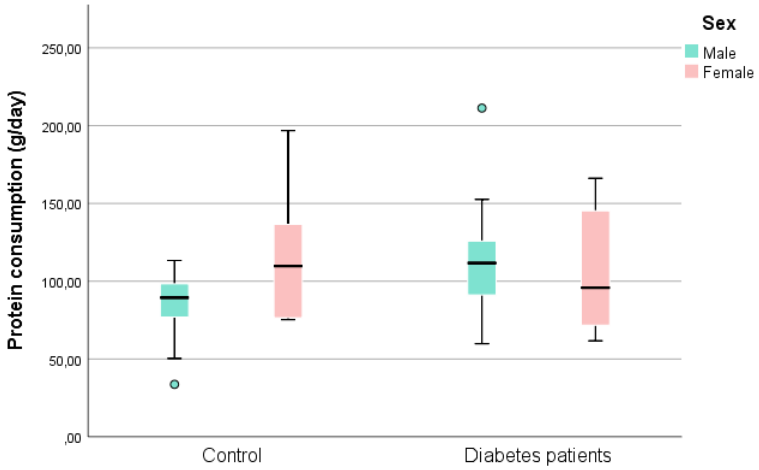
Supplementary Figure 29. Polyunsaturated fat consumption (g/day) in both diabetes patients and control groups.



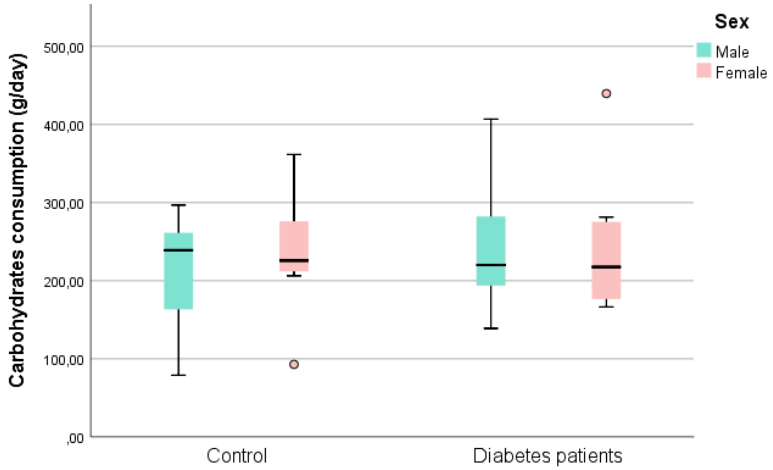
Supplementary Figure 30. Saturated fat consumption (g/day) in both diabetes patients and control groups.



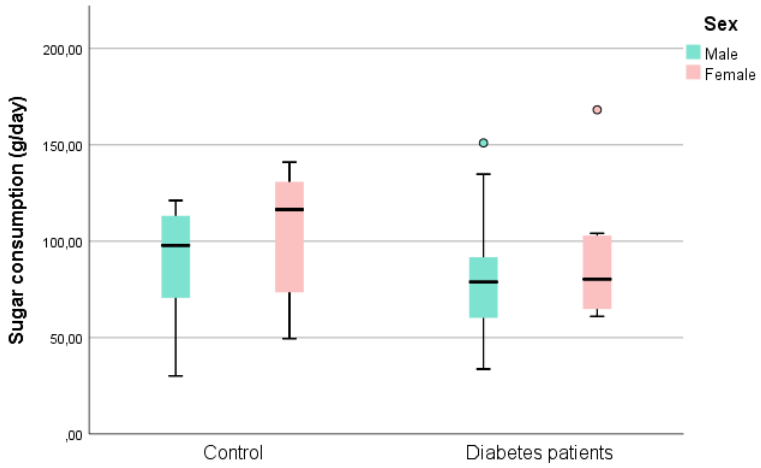
Supplementary Figure 31. Energy intake (Kcal/day) in both diabetes patients and control groups.



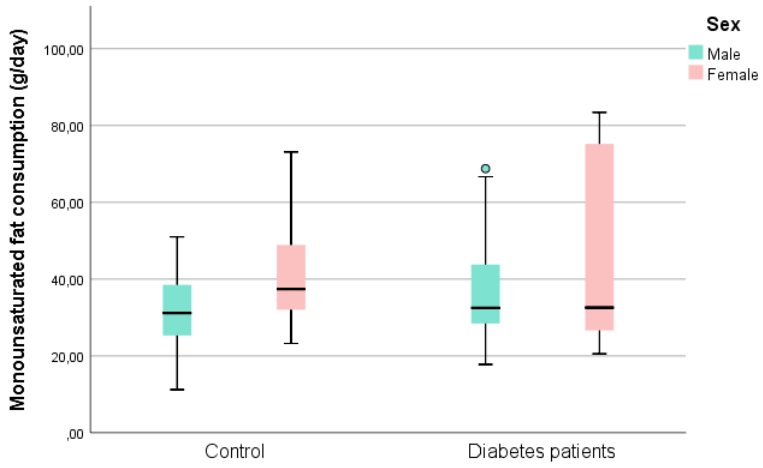
Supplementary Figure 32. Protein consumption (g/day) in both diabetes patients and control group. according to sex.



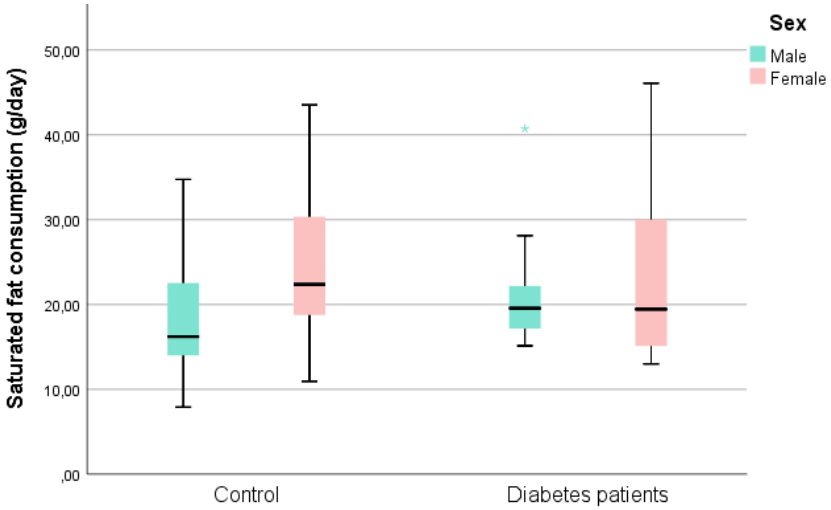
Supplementary Figure 33. Carbohydrates consumption (g/day) in both diabetes patients and control group. according to the sex.



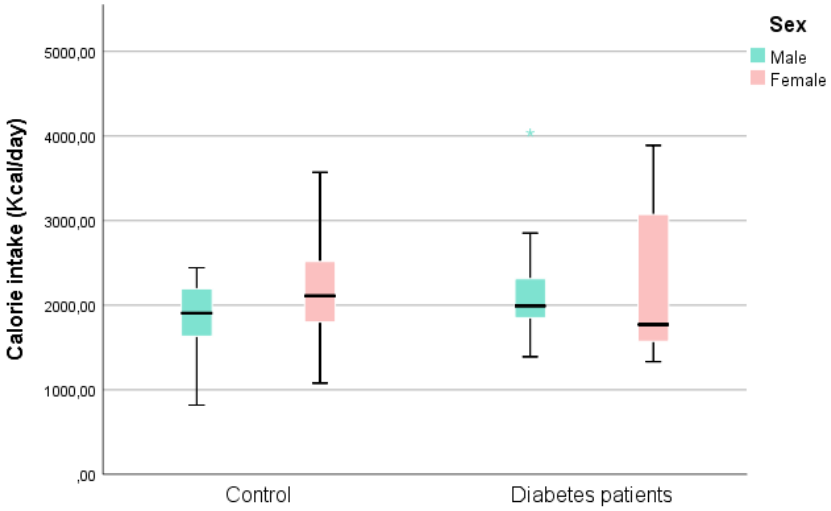
Supplementary Figure 34. Sugar consumption (g/day) in both diabetes patients and control group. according to the sex.



Supplementary Figure 35. Monounsaturated fat consumption (g/day) in both diabetes patients and control group. according to sex.



Supplementary Figure 36. Saturated fat consumption (g/day) in both diabetes patients and control group. according to the sex.



Supplementary Figure 37. Energy intake (Kcal/day) in both diabetes patients and control group. according to the sex