Characterization of tongue dorsum microbiome from wine tasters

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Resumo

O microbioma oral tem vindo a ser alvo de grande atenção nos últimos anos devido à sua elevada abundância e diversidade em microrganismos e o seu papel na saúde do indivíduo. A presença destes microrganismos é apenas reconhecida quando o equilíbrio hóspede-hospedeiro se perde, e a doença se manifesta. Estes têm um importante papel em funções como o metabolismo, digestão e pressão arterial. O microbioma do dorso da língua, é um dos nichos mais complexos do corpo humano, abrangendo cerca de um terço da população bacteriana na cavidade oral. O microbioma nasal ainda é pouco explorado, e embora exista pouco conhecimento acerca dos microrganismos residentes nas narinas, é conhecido que o olfacto tem um importante papel na perceção do sabor. Portanto, é fundamental o estudo da composição destes dois microbiomas e a sua relação com o sabor.

O objetivo principal desta investigação é descobrir quais as diferenças, se existirem, que ocorrem entre os microrganismos presentes no microbioma oral de provadores de vinho e não provadores. Para atingir este objetivo, o microbioma do dorso da língua e da narina de provadores de vinho e não provadores foi caracterizado através de metagenómica. A relação de outros fatores como os seus hábitos alimentares, estilos de vida e higiene oral, com a composição do microbioma foi considerada.

O microbioma oral de 29 provadores de vinho e 30 não provadores, e o microbioma nasal de 5 provadores de vinho e 5 não provadores, foi analisado através de sequenciação massiva da região V3-V4 do gene 16S rRNA. No caso dos provadores de vinho, foram recolhidas 2 amostras por indivíduo, sendo uma recolhida antes de uma prova de vinho e outra após a mesma (prov1 e prov2, respetivamente). Foi analisada a composição de ambos os microbiomas assim como a diversidade intra e interindividual. As medidas de diversidade utilizadas, foram a diversidade alfa (Índice de Shannon e Abundância de “sequências amplificadas”) e a diversidade beta (dissimilaridade de Bray-Curtis). O teste Permanova e análise de componentes principais permitiu comparar a composição do microbioma entre os dois grupos. Foram também exploradas as possíveis associações entre a constituição do microbioma do dorso da língua e nasal, e as diferentes variáveis como higiene oral, hábitos alimentares e estilo de vida.

As amostras relativas ao dorso da língua foram analisadas taxonomicamente através de quatro diferentes bases de dados (Greengenes, SILVA, RDP e HOMD), onde o maior

número de táxon identificada foi pela base de dados Greengenes, com um total de 55 táxons, 48 pertencentes ao grupo controlo e 38 ao grupo de provadores de vinho, dos quais 56% eram partilhadas entre os dois grupos, onde apenas foi possível identificar até ao nível da espécie, 5 táxon dos 55. A nível da diversidade alfa, apenas foi possível identificar diferenças ao nível da categoria “maior consumo de alimentos de sabor doce” e ao nível da diversidade beta, foi possível obter diferenças na composição do microbioma entre os grupos control, prov1 e prov2, quando agrupando control e prov1+prov2 e também entre o sexo feminino e masculino. Ao nível da classe, a maior diferença entre os grupos foi a presença do táxon *Bacteroidia* no grupo controlo, *Firmicutes* no grupo prov1 e *Bacilli* no prov2. Ao nível de género, registou-se uma maior abundância do táxon *Streptococcus* no grupo prov2 e *Veillonella* em ambos os grupos de provadores de vinho. As várias variáveis de dieta, estilos de vida e higiene oral mostraram ter uma influência na presença de alguns táxons no microbioma oral.

As amostras pertencentes às narinas, foram analisadas como no microbioma anterior pelas quatro bases de dados descritas, onde a base de dados Greengenes obteu novamente o melhor resultado na identificação de táxons, com um total de 22. Pertencendo ao grupo controlo 19 e ao grupo de provadores de vinho 12, onde 40% eram partilhadas entre os dois grupos e apenas duas foram identificadas até ao nível da espécie. Não foram encontradas diferenças significativas ao nível da diversidade alfa, beta, não tendo encontrado diferenças ao nível da composição do microbioma nasal entre os dois grupos. Todavia, foi possível identificar diferenças taxonómicas ao nível da classe e género. Onde, ao nível da classe se verificou uma maior abundância do táxon *Alphaproteobacteria* no grupo controlo e *Clostridia* nos provadores de vinho. Ao nível do género, registou-se uma maior abundância do táxon *Brachybacterium* no grupo controlo e de *N09* e *Peptoniphilus* no grupo de provadores de vinho. Tal como mencionado anteriormente, as mesmas variáveis foram testadas e verificou-se que certas categorias tinham uma influência na presença de alguns táxons no microbioma nasal.

Este foi o primeiro estudo nesta área de investigação, não existindo outros estudos que caracterizem o microbioma nasal, oral ou do dorso da língua de provadores de vinho portugueses.

Palavras-chave: microbioma, dorso da língua, nasal, metagenómica, provadores de vinho, sequenciação do gene 16S rRNA, higiene oral, dieta, perceção de sabor

Abstract

The oral microbiome has received major attention in recent years, due to its high abundance and diversity of microorganisms and its role in individual health. The presence of these microorganisms is only observed when the balance between host and guest vanished, and the disease manifests itself. These microorganisms play an essential role in functions such as metabolism, digestion or blood pressure. The tongue dorsum microbiome is one of the most complex niches in the human body, hosting approximately one-third of the bacterial population of the oral cavity. The nasal microbiome is relatively unexplored, and although there is still not much knowledge about the microorganisms residing in the nostril, it is known that olfaction plays an outstanding role in the perception of flavor. Therefore, it is essential to understand the composition of these two microbiomes and their relationship with taste.

The main aim of the present work is to find which differences, if any, occur between the microorganisms present in the oral microbiome of wine tasters and non-tasters. To achieve this goal, the tongue dorsum microbiome and nostril microbiome of wine tasters and non-tasters were investigated through metagenomics. The relationship of other factors as diet, oral hygiene and lifestyle habits with microbiome composition were considered.

The tongue dorsum microbiome of 29 wine tasters and 30 non-tasters and the nasal microbiome of 5 wine tasters and 5 non-tasters were analyzed through massive sequencing of the V3-V4 region of the 16S rRNA gene. Regarding wine tasters and oral samples, two samples were collected per individual, one collected before a wine tasting and the other after it (prov1 and prov2 respectively). The composition of both microbiomes was analyzed, as well as intra and inter-individual diversity. The diversity measures used were alpha diversity (Shannon index and Amplicon Sequence Variants abundance) and beta diversity (Bray-Curtis dissimilarity). The Permanova test and principal coordinate analysis allowed to compare the composition of the microbiome between the two groups. In addition, possible associations between the components of the dorsum tongue and nasal microbiome and different variables such as oral hygiene, diet and lifestyle were explored.

The tongue dorsum samples were analyzed taxonomically through four different databases (Greengenes, SILVA, RDP and HOMD), where the highest number of

identified taxa was obtained for Greengenes database, with a total of 55 taxa, 48 belonging to the control group and 38 to the wine tasters' group, sharing a 56% of taxa between both groups. It was only possible to identify 5 taxa out of the 55, at the species level. Concerning alpha diversity, the only significantly difference obtain was in the variable "consumption of sweet-related foods". At beta diversity level, it was possible to identify differences in the composition of the oral microbiome between the control, prov1 and prov2 groups, and also when grouping in control and prov1+prov2. The same analysis between genders indicated a difference in the composition of the oral microbiome between females and males. Differences were obtained at the class level between groups, with a higher abundance of the taxa *Bacteroidia* in the control group, *Firmicutes* in the prov1 group and *Bacilli* in the prov2 group. At the genus level, the higher abundance of the genus *Streptococcus* was obtained in the prov2 group, and *Veillonella* in both groups of wine tasters. The variables of diet, lifestyle and oral hygiene were shown to influence the presence of some taxa in the oral microbiome.

The nostril samples were also analyzed by using the four databases previously described, obtaining the best results for taxa identification with Greengenes database, with a total of 22 taxa, 19 present in the control group and 12 in the wine tasters' group. 40% of taxa were shared between both groups and only two taxa were identified up to the species level. No significant differences were found in the analysis with alpha or beta diversity, indicating no differences in the composition of the nasal microbiome between the two groups. However, it was possible to identify taxonomic differences at the level of class and genus. At the class level, there was a higher abundance of *Alphaproteobacteria* in the control group and *Clostridia* in the wine tasters' group. At the genus level, *Brachybacyerium* had a higher abundance in the control group and *N09* and *Peptoniphilus* in the wine tasters' group. As mentioned above, the same variables were analyzed and were found that certain categories had an influence on the presence of some taxa in the nasal microbiome.

This was the first study in this subject since there are no studies that characterize the nasal, the oral or the tongue dorsum microbiome of Portuguese wine tasters.

Keywords: microbiome, tongue dorsum, nasal, metagenomics, wine tasters, 16S rRNA gene sequencing, oral hygiene, diet, taste perception

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List of Abbreviations

PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
HOMD	Human Oral Microbiome Database
FISH	Florescent in Situ Hybridization
HMP	Human Microbiome Project
IHMC	International Human Microbiota Consortium
NGS	New Generation Sequencing
NIH	U.S National Institute of Health
16S rRNA	16s Ribosomal RNA
OTU	Operational Taxonomic Unit
PROP	6-n-Propylthiouracil
QIIME	Quantitative Insights Into Microbial Ecology
RNA	Ribonucleic acid
RDP	Ribosomal Database Project
SCFA	Short Chain Fatty Acids
ASVs	Amplicon Sequence Variants

1. INTRODUCTION

The community of microorganisms inhabiting a given environment is referred to as the microbiota, while the term microbiome is used to indicate microbiota collective genomes (Turnbaugh *et al.*, 2007). The Human body harbors trillions of microorganisms that create a complex ecosystem (Lloyd-Price *et al.*, 2016). Microorganisms of the human microbiome are estimated to out-number the host body cells by a factor of ten. These complex communities - normal residents of the skin, oral cavity, vaginal, and intestinal mucosa - oversee many essential physiological functions (Zaura *et al.*, 2009). The human microbiota is composed predominantly of bacterial populations (Petrosino *et al.*, 2009), which include commensal hosts necessary to maintain healthy tissues, playing several roles such as those underlying immunity response preparations insuring a prompt and efficient immune response (Devine *et al.*, 2015). But, often the presence of this microorganisms is only noticed when the balance between microbiota and host is lost, and a disease state possibly manifests itself (Devine *et al.*, 2015).

The diverse human tissues that support large microbial resident populations may, therefore, create a living interface delivering significant benefits for the host. However, while most of the evidence for benefits and homeostatic activities of the microbial residents derives from studies on intestine microbiota, the characterization of other body regions is now a very dynamic field that continuously have been providing information about the influence, role, and balance between microbiota and the host health (Almeida-Santos *et al.*, 2021; Devine *et al.*, 2015).

1.1. Microbiota Studies

The first evidence of bacteria from oral cavities was reported by Antony van Leeuwenhoek in the 16th century by using a microscope of his construction. This laid the foundations for the identification of microbes other than bacteria (Sharma *et al.*, 2018). But, it was only in the 19th century that Robert Koch and other pioneer scientists discovered the culture media and helped in preliminary studies with associations between microbes and infection diseases (Sharma *et al.*, 2018). They used nutrients in a solid phase like potato slices or gelatin to cultivate and isolate microorganisms, thus making their count and visualization possible (Escobar-Zepeda *et al.*, 2015). Later on, at the end of the 19th century, the Russian Microbiologist Sergei Winogradsky, came to the conclusion that microorganisms need special conditions to grow (Ackert, 2012), and this has greatly revolutionized microbiology.

For centuries, the study of microorganisms has been based on culture-dependent techniques that provided an insight into the microbial world but limited in resolution compared to other applications (Escobar-Zepeda *et al.*, 2015). Normally, in media-dependent microbiological studies, the most prevalent microorganisms are those that grow well in the lab environment, such as *Escherichia coli*. Therefore scientists started to suspect that many human-associated microbial species remained undiscovered (Lloyd-Price *et al.*, 2016). In the 1970s, with the advent of molecular biology, Carl Woese proposed the use of ribosomal RNA (rRNA) genes as molecular markers for life classification (Woese *et al.*, 1990), which produced a giant leap forward in the field of microbiology (Escobar-Zepeda *et al.*, 2015).

The development of DNA-based culture-independent methods emerged in the 1980s. These approaches are based on the analysis of DNA variation after extraction from a biological sample as opposed to individually cultured microbes, which permits to address several aspects of microbial communities, such as taxonomic diversity and functional studies (Morgan & Huttenhower, 2012). A couple decades later, a set of new molecular techniques that appeared at the end of the 20th century such as the polymerase chain reaction (PCR), rRNA genes cloning and sequencing, and Fluorescence I*n Situ* Hybridization (FISH), granted access to a "new uncultured world" of microbial communities. Yet, despite all these technical and conceptual molecular advances, there were questions that still remain unanswered, among which are those underlying the relationship between microbiome and environment (Escobar-Zepeda *et al.*, 2015).

The first study in which the 16S rRNA gene was used to characterize microbial communities without culturing dates to 1990, and consisted of the analysis of large microbial biomasses from oligotrophic waters (Giovannoni *et al.*, 1990). Some years later, Handelsman *et al.* (1998), accessed the genomes of soil microorganisms using a DNA template directedly isolated from whole soil-content samples. This study pioneered a new era on microorganism genetics referred to as metagenomics. This term results from the combination of the Greek word, *meta,* which means "transcendent", with the term *genomics*, which means a collection of genetic material. Thus, metagenomics entails the study of a collection of genetic material from an uncultivated and cultivated mixed community of organisms (for a deep understanding see National Research Council (US) Committee on Metagenomics, 2007). Metagenomics is therefore a very new research field that is presently harnessed with Next Generation Sequencing (NGS), enabling the massively parallel sequencing of the genetic material (DNA/RNA) of an entire microbial community. However, metagenomics can also be employed in functional studies on how genes from those microorganisms translate in proteins and produce

different phenotypes under the influence of different environments, thus affecting a given microbiome (Wang *et al.*, 2015).

The development of low-cost NGS sequencing platforms generating millions of DNA molecules with different yields and lengths (Escobar-Zepeda *et al.*, 2015). NGS encompasses a suite of techniques that revolutionized genomic research. Due to its sensibility to low DNA template quantity and selectivity, NGS can therefore be used to identify variants that reside in just a few per cent of the cells, including mosaic variation (Behjati & Tarpey, 2013). The first of NGS platforms that revolutionized the genomics and metagenomics areas was the 454 sequencer or "pyrosequencing" (Escobar-Zepeda *et al.*, 2015). It was this technology that permitted the complete genomes of two bacteria in 2005, obtained in a single run of their Genome Sequencer (Margulies *et al.*, 2005). In 2006, the first Solexa sequencer was launched to be acquired by Illumina in 2007 under the name Illumina Genome Analyzer. This system relies on a sequencing-by-synthesis approach in which all four nucleotides are added simultaneously to the flow cell channels along with DNA polymerase (Mardis, 2008). The recent arrival of two new sequencing-technologies, which solve part of the biases of those already existing, opening the era of Third-generation sequencing. Those latest technologies are PacBio RS from Pacific Bioscience and Oxford Nanopore (Escobar-Zepeda *et al.*, 2015).

The two main approaches to study microbial community diversity are the amplicon-sequencing of 16S ribosomal RNA (16S rRNA) and shotgun sequencing, which is based on producing random fragments of DNA that can be sequenced individually and then reassembled by computers into their original order, based on overlapping ends (Ranjan *et al.*, 2016). PCR-based methods using universal primers assure rapid bacteria detection by using nucleic acids extracted directly from biological samples but may fail to amplify all clades with the same efficiency (Ranjan *et al.*, 2016). On the other hand, shotgun sequencing bears considerable advantages, as it can more accurately identify the lower taxonomic levels (i.e., species, sub-species), although it is a more expensive method and requires a heavier computational power for data analysis (Ranjan *et al.*, 2016).

The amplicon sequencing of 16S rRNA is the most used approach. Since it relies on a simple PCR amplification and primers that recognize highly conserved regions of the target gene. The 16s rRNA gene consists of nine hypervariable regions (V1-V9) separated by the other ten which are highly conserved (Figure 1).

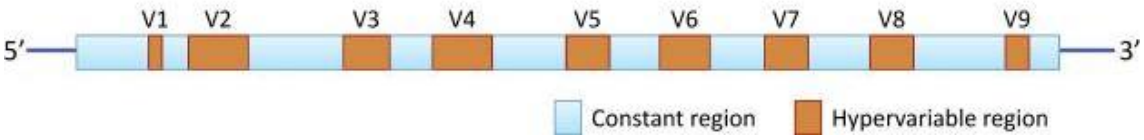

Figure 1. 16s rRNA gene, conserved and hypervariable regions (adapted from Sarangi et al., 2019)

Due to constraints of the sequencing technology employed, the 16S rRNA gene is sequenced only partially. This poses some challenges for the selection of specific primers, a key step to study bacterial phylogeny across multiple environments (Yang *et al.*, 2016). A major advantage of 16S rRNA analysis is the genetic information on millions of species available in public databases as opposed to other types of genomic databases, which contain tens of thousands (Breitwieser *et al.*, 2019). A key factor on the outcome of the BLAST search is the choice of the database. Reference databases contain several thousand 16S-rRNA gene sequences, with information on the bacterium from each one was obtained. Several databases are available as SILVA, Ribosomal Database Project (RDP), Greengenes, EzTaxon-e, and RNACentral (Yilmaz *et al.*, 2014). The importance of this topic has been the main driver in the development of new methods, protocols to generate new information have been addressing many questions, which the answers have been helping to fill the knowledge gap on the complexity of the microbial communities (Caselli *et al.*, 2020).

1.2. The Oral Microbiome

The human body includes different microhabitats inhabited by a variety of microorganisms, of which some are host specific. The success in the NGS characterization of the human microbiome converged in the Human Microbiome Initiative in the U.S National Institute of Health (NIH) Road which was roadmap programs extended to all areas of health and disease research, one of the largest cohort studies carried out so far (Mardis, 2008). The first investigations on oral microbiome based on 16S rRNA gene amplicon sequencing date from a decade ago (Zaura *et al.*, 2009). Based on the Human Oral Microbiome Database (HOMD), more than 750 prokaryote species have been already identified in the oral cavity, making the oral microbiota the second most abundant microbiota after the gastrointestinal one (Verma *et al.*, 2018). The oral cavity is a complex habitat with hundreds of microbial species and, even though bacteria are often considered the most common microorganisms (Esteban-Fernández *et al.*, 2017), several species of fungi, viruses, and protozoa also populate the oral cavity (Caselli *et al.*, 2020). To clarify, bacteria are prokaryotes with a nucleoid rather than an enveloped nucleus (Salton & Kim, 1996).

Bacteria are classified into two large categories according to the type of cell wall: gram-positive and gram-negative. The first do not present lipopolysaccharide layer encasing the cell, presenting thick walls composed of peptidoglycans (Dertz & Raymond, 2003). Conversely, the cells of the latter are characterized by two layers, of which the outer is composed of lipopolysaccharide (Sandhir, 2014). Variation in the wall structure dictates the environments where these two types of bacteria occur. Gram-positive bacteria are capable of survival and growth in less dilute systems compared to gram-negative which prefer low osmolarity environments, mostly growing in water and dilute solutions (Sandle, 2016).

Sequenced data of the resident microorganisms in oral microbiota are available in specific microbial databases of organs, realized thanks to the effort of the NIH, the International Human Microbiota Consortium (IHMC), and the Human Microbiome Project (HMP). The microbial diversity of the oral cavity has been widely studied mostly through the characterization of 16S rRNA hypervariable regions V1, V2, V3, and V4, chosen in view of their high discriminatory power (Verma *et al.*, 2018).

The oral cavity is a complex environment made of several ecological niches over intraoral surfaces made of both soft and hard tissues (Zaura *et al.*, 2009). The knowledge of the resident microbiota, inhabiting about 80% of the oral cavity surface, is still very limited. Moreover, the understanding of the composition of the oral soft tissue is limited compared to the dental plaque, for which the higher number of studies produced relied on a variety of subjects, samples, and species examined with faster methods (Mager *et al.*, 2003). However, the oral microbiome is involved in a number of essential functions spanning from the maintenance of systemic and oral health to the affection of the psychological and nutritional level as well as that of the individual immune system (Hall *et al.*, 2017).

Interindividual differences in the oral environment occur as a result of the quality and quantity of saliva, social habits, tobacco consumption, diet, medicine exposure, hormonal fluctuation, and variability in levels of host defense (Hall *et al.*, 2017). The oral microbiome influences the appearance or progression of oral diseases when shifts in the balance of bacterial composition turn into pathogenic effects (Rabe *et al.*, 2019). Most of the existing studies focus on the microbiome in the state of disease, without a specific map of the microorganisms present in a healthy condition (Caselli *et al.*, 2020). Therefore, it would be advisable to assess the composition of a healthy microbiome before referring to it as causing diseases or disease-related alterations (Rabe *et al.*, 2019).

1.2.1 Bacterial Community

The human oral cavity hosts a large community of bacteria, most of which provide significant benefits. Commensal bacteria play a role in this homeostasis modulating and regulating the host in several mechanisms (Devine *et al.*, 2015). Yet, the knowledge about oral ecology and the mechanisms that maintain the balance between microbiota and host needs to be deepened. For example, it was possible to isolate immunosuppressor oral bacteria, which represent about 30% of the resident streptococci in the plaque or tongue. In the mouth, neutrophils are key to protect gingival tissue, and resident bacteria in the subgingival plaque can influence their development by regulating low levels of expression of intracellular adhesion molecules (Devine *et al.*, 2015).

It is well known that the composition of the bacterial community varies in different parts of the oral cavity. Molecular-based techniques like 16S rRNA profiling provide new insights on the diversity of the oral cavity microbiome (Simón-Soro *et al.*, 2013). The microorganisms inhaled and/or ingested have a large influence on the oral cavity, but only 54% of the bacteria species of the local microbiome are cultivable and identifiable, 14% are cultivable but not identifiable, and the remain 32% cannot be cultivated at all (Caselli *et al.*, 2020).

The reason underlying the high variability of the bacterial composition might reside in the physic and chemical gradients in different areas of the mouth. Oxygen can be considered one of the main environmental factors affecting bacterial distribution. A plethora of studies found extensive evidence of differences in the abundance of obligate aerobes, such as *Fusobacterium*, on the tongue, as opposed to aerobes and facultative anaerobes such as *Streptococci* in vestibular sites or on incisors and canines, which are exposed to higher oxygen levels. It is also reported that the pH has an important role in the distribution pattern, with vestibular and lingual surfaces playing a different buffering effect of saliva (Simón-Soro *et al.*, 2013). According to Wang *et al.* (2019), the oral microbiome also presents significant differences between populations across countries, which can be explained by the interplay of surrounding environment, lifestyle and food habits.

A large number of operational taxonomic units (OTU) is another factor of complexity. In a healthy microbiome, it is possible to isolate over 3600 unique sequences assigned to 500 OTU in the oral cavity of a single individual. The most abundant taxa belonged to *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes* and *Fusobacteria* (Zaura *et al.*, 2009). More recently, Caselli *et al.* (2020) considered that the bacterial genera more frequently associated with healthy oral microbiome were *Streptococcus, Granulicatella, Neisseria, Haemophilus, Corynebacterium, Rothia, Actinomyces, Prevotella,*

Capnocytophaga, Porphyromonas, and *Fusobacterium*. However, bacterial diversity also varies according to the region of the oral cavity, with saliva and tongue microbiome hosting the highest diversity, dominated by genera *Rothia, Prevotella, Streptococcus, Veillonella, Fusobacterium, Neisseria,* and *Haemophilus* (Rabe *et al.*, 2019). Moreover, the oral microbiome composition varies between different microhabitats and each individual holds its own "microbial identity" (Caselli *et al.*, 2020). Thus, there is much room to fill the knowledge gap about the composition of a healthy oral microbiome as opposed to that of a human microbiome in a state of disease.

1.2.2 Tongue Dorsum Biofilm

The microbiota of the tongue dorsum is one of the most complex niches in the human body, hosting approximately one-third of the bacterial populations of the oral cavity and acting as a pathogen reservoir for infection or reinfection of the supragingival and subgingival plaque (Faveri *et al.*, 2006).

The oral bacteria community is structured in multi-layered structures, called "biofilms", where commensal microorganisms and pathogens are embedded in a self-excreted matrix of polymeric extracellular substances. The formation of the biofilm occurs in two steps. First, the new colonizers get attached to the oral surface, originating a microbial monolayer. Second, these new hosts migrate, while secondary and late colonizers sequentially establish new colonies, which leads to a maturated biofilm (Esteban-Fernández *et al.*, 2017). Inside of these layers, the bacterial species can develop synergetic or antagonist relations among them and other microbial taxa (Esteban-Fernández *et al.*, 2017). These microorganisms have distinct receptors and adhesion molecules that can dictate the development of different biofilms on the oral surface (Faveri *et al.*, 2006).

The tongue coating consists of the material adhering to the tongue surface and characterized by a rich microbial community as opposed to the mucosal pellicle (Neyraud & Morzel, 2019). In a situation of poor or no oral hygiene as opposed to regular oral care, its composition is dominated by gram-negative species along with some periodontal pathogens (Faveri *et al.*, 2006). It is also known that after a period of scarce oral hygiene, there is an accumulation of microorganisms of which many are pathogens. This is probably due to the morphology of the tongue dorsum, which presents various irregularities representing a favorable environment for microorganism accumulation, with anatomic niches where microorganisms are well protected from the flushing action of the saliva. In such situations, the low oxygen levels promote the development of anaerobic microbiota (Faveri *et al.*, 2006). Therefore, the tongue can be considered a

microorganism' reservoir that deeply affects saliva microbiome composition, suggesting that more attention should be paid to the tongue and oral hygiene (Rabe *et al.*, 2019).

In recent years, the analysis of the tongue microbiome has been gaining more attention as clinical assessments (i.e., diagnostics) based on traditional Chinese medicine promoted association studies in health sciences (Rabe *et al.*, 2019). The tongue dorsum carries a distinctive ecological niche by providing a large surface that helps to promote the acquisition of microorganisms along with food debris, saliva, and degseraenerated epithelial cells which can be responsible for the metabolism and growth of the microorganisms (Dwivedi *et al.*, 2019). Several studies about the characterization of microorganisms harbored by the human dorsum tongue found a predominance of aerobic organisms or facultative anaerobes, like *Firmicutes* and *Bacteroidetes*. NGS-based studies have evidenced that the most abundant taxa in the tongue coating microbiome of healthy individuals were *Firmicutes*, *Proteobacteria,* and *Bacteroidetes* (Neyraud & Morzel, 2019).

1.3. Brief History of Wine Tasting

For a better understanding of what is a wine taster the meaning of some terms will be clarified first:

- The *Winemaker* is the person in charge of the process of making the wine.

- The *Oenologist* the one responsible for choosing and applying the best technique to use in all the phases of vinification and interpreting the laboratory results of the obtained broths.

- The *Wine taster* is a critic, writer, buyer, or any other professional who tests the quality of wine by tasting.

- The *Sommelier* works with wine selection in relation to final consumers at restaurants, hotels, and bars.

The Sommelier is also responsible for tasting events, demonstrations, and wine production courses (CVRPS, 2021). This profession officially debuted in 18th century France under the French Revolution, but it appeared long before in association with the ritual of tasting the wine before it was served to check if it had not been adulterated. Over time, sommeliers started to serve as a guarantee that the product was genuine (Pereira, 2017) and wine tasting started to be a fixed step of wine production even prior to the consumption of the final product in contact with consumers. According to the American Society of Testing Materials, a wine expert is someone (often operating alone) with extensive experience in different wine categories performing perceptual evaluations

about the effects of variations in raw materials, processing, storage, and aging (ASTM, 2005). Based on the previous definition a wine expert can be associated with the term sommelier, oenologist, or wine taster. In Portugal as in all Europe, there are regional wine commissions as a process of control. These commissions have chambers of tasters (composed of about 20 professionals) to do organoleptic tests of the different wines from each region. To become part of these panels, the individuals are selected carefully and trained for several months. These trainer panels started to be developed in the first half of the 20th century, which also witnessed the emergence of sensory evaluation. They assured greater objectivity to the measurements of wine analysis by integrating the subjectivity and variability intrinsic to human evaluators (Pinto, 2021).

Wine production in Portugal dates to the second millennian before common era (B.C.) when the first vineyards were planted along the Tejo and Sado river valleys. It is thought that the Phoenicians (10th century B.C.), the Greeks (7th century B.C.), and Celts (6th century B.C.) had a preeminent role in the habit of wine consumption. Later, the Romanization process consolidated wine production in Portugal, as it was up to the Roman colonies to satisfy the demand and ensure that wine flowed abundantly, throughout the Empire. The Germanic tribes that took over the Roman Empire by the 8th century B.C. also adopted wine consumption. The 15th and 16th centuries defined a decisive evolution in the history of wine in Portugal, with, Lisbon becoming the largest center of consumption and distribution of wine throughout the Portuguese Empire (Rocha, 2018).

The evaluation of wine in Portugal was traditionally assigned to one or a group of winemakers, but since the late 1980s, the use of sensory evaluation techniques increased, especially under the leadership of Ann Noble at the University of California (Lesschaeve, 2007). In addition to winemakers, others began to be responsible for evaluating wine quality, including wine writers and critics, sommeliers, and wine merchants (Langstaff, 2010).

1.4. Oral microbiome and taste perception

The relationship between the oral microbiome and taste perception has been the main subject of several studies. A recent study about the association between the oral microbiome and taste perception evidenced that the latter could have an important role in diet and body energy balance (Cattaneo *et al.,* 2019). It was also found a relationship between the reduction of the taste perception and an increase in specific oral bacteria. The response to PROP (6-n-Propylthiouracil) was evaluated, revealing that subjects with

greater responsiveness to PROP presented differences in the relative abundance of certain bacteria compared to subjects less responsive to PROP. The group more responsive to PROP presented a greater abundance of the gram-positive bacteria belonging to genera *Actinomyces, Oribacterium, Solobacterium,* and *Catonella,* as well as to gram-negative such as *Campylobacter*. Further correlations were found, between sensitivity to salty and sour with specific bacteria. Greater sensitivity for these tastes was significantly associated with the presence of bacteria from the order of *Clostridiales* and *Bacteroidales*. In this respect, Takahashi (2015) explained this association based on the bacterial ability to degrade carbohydrates in disaccharides, monosaccharides, and organic acids, which are used as “building material” for biofilms.

In a study, Pickering *et al.* (2013) evaluated the responsiveness to PROP and if it contributed to differences in the individual orosensory perception (i.e., aroma, taste, image, and/or texture) and the hedonic responses for food and beverages applied to wine specialists and foodies (name used to define gastronomes). It was found that a higher response to PROP is related to a higher oral stimulation, leading to speculate that gastronomes are represented as PROP super-tasters. The evidence of the strong relationship between gene and environment in terms of taste detection capabilities was found in wine experts and attributed to the occurrence of a stronger innate taste. This correlation is based on the hypothesis that individuals can self-select for some interests and professions. Therefore, individuals with a greater connection and involvement with food and beverages differ from others in their ability to respond to PROP (Pickering *et al.*, 2013).

The tongue biofilm also plays a fundamental role in affecting the sensory and oral perception, as it generates abundant compounds, some of which interfere with the metabolic activity of the oral microbiome. Moreover, it is suggested that glycine, valine, and leucine amino acids have the potential to change sensory perception (Gardner *et al.*, 2020). Thus, the correct understanding of the role played by the tongue coating in the taste perception requires the profiling of microbiota from different sites of the oral cavity and individuals. For example, an increase in the recognition of salty and sour tastes was observed when elderly individuals are subjected to tongue brushing (Neyraud & Morzel, 2019). This sensorial sensitivity change is explained by the coating that blocks the taste pores. Therefore, oral bacteria are tightly related to taste perception, either by the direct influence of the diet or indirectly by the synthesis of sensory-active molecules. In this respect, the tongue dorsum bacterial metabolism can enrich or deplete the medium close to the taste receptors, having an impact on taste perception through the phenomenon of sensory adaption (Neyraud & Morzel, 2019).

Even if a large number of studies have found significant associations between tongue coating and taste perception, Feng *et al.* (2018) proposed that the bacterial component of the coating deserves more attention, as the natural material found on its surface is still poorly described unless it exceeds normal amounts, for example in patients suffering from halitosis. An increased level of sucrose in the tongue coating can indicate a lower proportion of bacteria capable of converting it into glucan and fructan, causing less firmness in the biofilm structure. In other words, the physical barrier between gustative molecules and taste receptors would be less efficient and the sensitivity increased, but this proposition needs to be assessed.

Regarding the association between oral microbiota and wine taste perception, a study conducted in Italy by Frumento (2018) revealed that the latter can fluctuate across individuals due to differences in taste perception, olfactory sensitivity, allergies, smoking habits, and sensory memory. It was also observed that saliva can cause changes in wine molecules through enzymes that modify the composition of the oral microbiota itself. Thus, its role in the synthesis of these salivary enzymes is of paramount importance, and the absence of the bacteria necessary to produce them may change the individual perception of wine molecules (Frumento, 2018).

1.5. The Key Influencers of the Oral Microbiome

1.5.1. Oral Hygiene

Oral hygiene plays an important role in microbiome modulation. The characteristics of the dorsal surface of the tongue are responsible for the persistence of a high bacterial load. The anterior part of the tongue is rougher due to the presence of numerous papillae with different structures and functions.

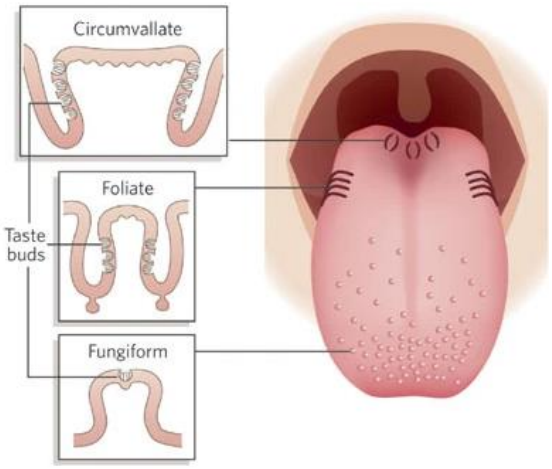

Figure 2. Anatomical location of the taste buds in the tongue dorsum (J. Chandrashekar et al., 2006)

On the other hand, lacking tongue hygiene can increase the bacteria load and alter its taxonomic composition, with an increase of anaerobes. Experimental data evidenced that up to 18 species of bacteria, pathogens included, occur in greater abundance after a period without proper oral hygienization, suggesting that the tongue surface is an important reservoir of pathogens for oral disease (Faveri *et al.*, 2006).

1.5.2. Food Habits

As mentioned earlier, the oral cavity is considered a complex community made of symbionts whose pathogenic potential is unleashed only when the balance between microbiota and host is lost. Humans and parasites coevolved over the past thousand years, but their relationship has been affected also by external factors like environmental changes and social norms. Investigations comparing ancient and recent dental plaques revealed an evolution on the oral microbiome toward a carcinogenic configuration due to the hunter-gatherer transition to agricultural lifestyles and the industrial revolution (Kato *et al.*, 2017).

According to an exploratory study, associations between dietetic habits and human oral microbiome, in where it was possible to observe correlations between higher intake of glycaemic load and abundance of *Lactobacillaceae.* Individuals with higher levels of carbohydrates intake in the diet demonstrated an increase in the abundance of *Lactobacilli* and *Streptococcus mutans* in the oral cavity. A correlation was also found between the intake of short-chain fatty acids (SCFA) and Vitamin C in affecting the composition of the oral microbiome, which suggests a positive relation between SCFA and relative abundance of *Betaproteobacteria* and *Fusobacteria* as well as between vitamin C and the occurrence of *Fusobacteria Leptotrichiacceae* and *Lachnospiracceae* (Kato *et al.*, 2017).

The relationships among taste perception, oral microbiota composition, diet, and food intake have been explored by Cattaneo *et al.* (2019), who demonstrated differences in taste perception associated with alteration of the usual food consumption regime. The authors reported that individuals with the lowest sensitivity for salty taste - referred to as hyposensitive group - consumed more frequently salty flour-based baked food products, saturated-fat-rich products, and soft drinks, than individuals with a higher sensitivity for salty taste, referred to as the hypersensitive group. Also, people with lower sensitivity to sweet taste consumed more sweets and desserts compared to the hypersensitive group.

Finally, subjects with higher sensitivity to bitter taste showed lower total energy values and carbohydrates intake. These differences in taste perception play an important role in influencing the diet. For instance, individuals with reduced perceptive capabilities were also those associated with an excessive food intake responsible for an increased taste stimulation which leads to less healthy food choices. Moreover, alterations of the oral microbiota diversity have also been observed in association with higher sensitivity to salty and sour taste which, in turn, is related to the abundance of *Clostridiales* and *Bacteroides*. Specifically, an increase of *Clostridis* abundance is often associated with a higher energy value diet based on fat and protein. On the contrary, bacteria presence was inversely related to fiber consumption. Thus, the oral microbiota is conceived as a reliable marker of less healthy habits (Cattaneo *et al.*, 2019).

Beyond alcohol, the oral microbiome is also influenced by tobacco consumption. Chronic users of both were studied to evaluate if tobacco has an impact on the growth of certain bacterial species, with special reference to altering the normal abundance of those regularly found in the mucosa of the oral cavity. Collected data indicated that tobacco consumption, combined or not with alcohol, affects the oral microbiota, leading to a reduction in species richness. It would be important to conduct further studies in which subjects suffering from chronic alcohol consumption, but using no tobacco, are included, thus assessing the individual effect of these substances on the oral microbiome (Thomas *et al.*, 2014).

1.5.3. Wine consumption

Polyphenols are secondary plant metabolites that are found in grapes and red wine. Up to date, it was possible to verify that phenolic compounds are capable of selectively modulate the growth rate of susceptible microorganisms. Moderate consumption of wine can alter the composition of oral microbiota (Requena *et al.*, 2010). However, this conclusion relies on the limited information available concerning the effect of daily habits and regular consumption of red wine on the oral microbiota (Barroso *et al.*, 2015). Nonetheless, it is known that dietary polyphenols have health benefits, including antioxidant properties, anti-proliferative action, anti-inflammatory effect, anti-allergic, anti-hypertensive, and anti-thrombotic activity (Castaldo *et al.*, 2019; Golan *et al.*, 2019; Roman *et al.*, 2019), having also positive effects on microbiota composition (Kumar Singh *et al.*, 2019) and functionality (Mattos *et al.*, 2017).

During the last decade, several studies have shown that wine polyphenols and oenological extracts mainly derived from red wine and grape seed by-products are effective antimicrobials against certain bacterial species (Esteban-Fernández *et al.*,

2017; Le Roy *et al.*, 2020). It was also found that different drinking habits of red wine affect the diversity and existence of different groups of human saliva bacteria, but their medium is not destabilized by regular or moderate wine consumption (Le Roy *et al.*, 2020). Also the phenolic extracts of grapes, wine, and pomace are able to inhibit the growth of different *Streptococcus* strains associated with dental caries and an inhibitory effect on the selective growth of *Actinomyces oris, Fusobacterium nucleatum, Streptococcus oralis, Streptococcus mutans,* and *Veillonella dispar* in a biofilm model (Muñoz-González *et al.*, 2014). Other studies went deeper into the analysis of the microbiota composition at the different oral regions in individuals with different drinking habits and found that the oral plaque had a lower bacterial diversity in wine consumers compared to water consumers (Signoretto *et al.*, 2010).

1.5.4. The profile of the "Portuguese" oral microbiome

The study of the human microbiome composition has started to gain attention only over the last decade, and only a few studies have explored this topic in individuals from Portugal. A recent investigation characterized the oral microbiome of individuals with type 2 diabetes mellitus, reporting a higher number of taxa in the oral microbiome of the control group compared to the diabetics. The results of the study suggested that the relationship between the oral microbiome and diabetes is mostly related to lifestyle or the degeneration of the disease rather than to just having diabetes (Almeida-Santos *et al.*, 2021). Another study, focused on the oral fungal microbiota in smokers and non-smokers, concluding that tobacco smoking may alter the oral mycobiota and provide an environment of colonization of yeasts and pathogenic molds. The subjects of this investigation were students from the fifth year of the master degree of the Faculty of Dental Medicine of Porto University (Monteiro-da-Silva *et al.*, 2013).

Most of the studies carried out in Portugal are focus on the intestinal microbiota, being evident that there is a general lack of knowledge about the composition of the microbiome in healthy subjects of the Portuguese population, which makes the present study – focused on wine tasters – a pioneer study to fill this gap.

Overall, current knowledge on oral microbiota, and specifically those of the dorsum tongue, is still limited. Moreover, most of the information available was acquired through studies made before the advent of NGS and metagenomics. The fast-increasing body of research now addressing oral microbiota composition is very promising and bears a huge potential to bettering our understanding of aspects that go beyond disease all the way to taste perception. However, the fulfillment of these goals requires detailed information on the environment, lifestyle, diet, and oral and body health. Only by controlling those

factors, it will be possible to address questions regarding the association between the composition of dorsum tongue microbiota and the wine taster profession.

1.6. The Nasal Microbiome

The nose is an important site of pathogen colonization and still, is a microbiota relatively unexplored. The outermost segment of the nose, the nostrils, is a transition zone from the skin to the nasal cavity. Like the skin, the nostrils accommodate sebaceous glands, sweat glands, and hairs, and help filter inhaled air. Most of the knowledge on the nasal microbiota has been generated via cultivation, which showed that the most frequent phyla of the bacteria from the nostrils are mainly Firmicutes and Actinobacteria. At a lower taxonomic level, this region is known to harbor bacteria from the genera *Corybacterium, Propionibacterium,* and *Staphylococcus*, including the important pathogen *Staphylococcus aureus*. But, unfortunately most of the research on the nasal microbiome have been focused on one or a few pathogenic taxa (Lemon *et al.*, 2010).

A study conducted in subjects with exacerbated asthma, non-exacerbated asthma found that relative to a control group the nasal microbiome of subjects with asthma were enriched with taxa from Bacteroidetes and Proteobacteria. Giving the possibility that the nasal microbiota could have a role in asthma pathology and help to monitor the disease (Fazlollahi *et al.*, 2018). The understanding of the composition of the complex bacterial communities in which pathogens reside will provide new insights into why only some individuals become colonized with pathogens (Lemon *et al.*, 2010).

1.6.1. Taste and smell perception

There is a connection between taste and smell, and when we talk about the perception of flavor that becomes more obvious. Because when we “taste” the drink or food, it touches our mouths and senses its odor, via retronasal olfaction (Small & Green, 2012). About 75 and 95% of what we think comes from taste comes from the sense of smell (Spence, 2015). It is the olfactory component of food that is required for flavor identification, during the acts of chewing and swallowing it is released volatile molecules into the oral cavity that are conveyed to the brain by distinct pathways. The perception of flavor depends on multiple factors, and the interactions that lead to this act are still unknown (Small & Green, 2012). The fact that olfaction has an important role in the tasting of food inspired modernist chefs to deliver to their customers’ multisensory flavor experiences (Spence, 2015).

In this study, the main aim is to find which differences, if any, occur between the microorganisms present in the microbiota of tongue dorsum in wine tasters as opposed

to non-tasters. As a secondary aim, we investigate the nostril microbiome composition of wine tasters and non-tasters. By correlating variables, we expect to elucidate which are the main drivers affecting oral microbiome composition and achieve a better understanding of this complex and fascinating world.

2. OBJECTIVES

2.1. Main Objective

How the profession of wine taster modulates the oral microbiome composition and/or vice-versa yet remains unclear. Even if only a few studies relate oral microbiota with taste perception, they only focus on the enzyme expression and taste receptors. In this thesis, we will focus on the differences between the microorganisms present in the microbiota of the tongue dorsum in wine tasters as opposed to non-tasters and explore correlations between different lifestyles and dietary habits to have a complete understanding of the influence of these variables in the oral microbiome. By pursuing this approach, we propose to explore the possible correlation between microbiome composition and taste perception with a special focus on evidence of self-selection induced by sensory adaptation in people who are in frequent contact with the wine. For this, we will implement an exploratory and analytical investigation based on metagenomics to characterize oral microbiomes in wine tasters.

2.1.1. Ancillary Objectives

The nasal microbiome is still poorly described and studied, with the collection of samples from the nostril of individuals from a group of wine tasters and non-wine tasters we expect to see differences in the nostril microbiome of these groups by using a metagenomics approach. Across the entire career of a wine taster, it is necessary to capture the flavors of the wine, and thus it is necessary to train and develop an adequate olfactory perception. Considering that olfaction plays an important role in wine tasting we (1) proposed to test for differences in the microbiome present in the nostrils, and (2) test whether there is a significative association between high frequency of contact of the nostrils with the molecules present in wine and the microbiome profile.

2.2. Specific Objectives

To achieve the main aim and the auxiliary objectives, the following objectives were proposed:

- Characterization of the tongue-dorsum and nasal microbiome by identifying the bacterial species hosted by each individual, as well as their abundance;
- Compare tongue-dorsum and nasal microbiome of each group to identify site-specific taxa;
- Analyze of the bacterial diversity measures at the individual (alpha diversity), and the inter-individual (beta diversity) levels;

- Compare the tongue-dorsum and nasal microbiome profiles and diversity patterns in both wine tasters and the control group;
- Test whether the wine taster group possess any host-specific taxa which are not found in the control group or vice versa;
- Evaluate the impact of dietary, oral hygiene, and lifestyle on the tongue-dorsum microbiome profiles;
- test for an association between regularity of tasting, type of wine, preparation for the tasting process, and life habits influence the tongue-dorsum microbiome profile.

3. MATERIAL AND METHODS

3.1. Subject Selection

Samples were collected from a group of 29 wine tasters (age range 25 to 66; average age 45), composed by 7 women and 22 men recruited through different contacts, and 30 individuals (age range 30 to 54; average age 40) belonging to the control group including 17 women and 13 men. Our sample included a panel of wine tasters from the Bairrada Viticulture Commission, from Vila Real (Trás-os-Montes; Northern Portugal), and oenologists and sommeliers from Coimbra (Beira Litoral; central Portugal). All samples were taken from volunteers after reading and approved the informed consent (Supplementary document 1). Volunteers of both groups that had been under the influence of antibiotics over the last month prior to the survey were considered ineligible. This study was approved by the Bioethic Committee of CIBIO/InBIO (University of Porto).

3.2. Sample Collection and Questionnaire Administration

All the participants had to sign an informed consent stating all the relevant information about the study (Supplementary document 1). Due to the ongoing COVID-19 pandemic, sample collection had to be performed by the volunteers themselves, and the questionnaire was filled online via google forms (Supplementary document 2). The questionnaire aimed to retrieve information about food habits related to specific taste preferences (sweet, sour, astringent, and bitter), lifestyle (including smoking habits and alcohol consumption), oral hygiene, and, in the case of wine tasters, the preparation before tasting events and some aspects of the profession routine.

Participants were requested not to drink, eat, smoke, brushing teeth, and chewing gum for at least two hours before sampling. They were also provided with a step-by-step guide on how to perform sample collection (Supplementary document 3). The participants used swabs (SK-2S Isohelix Swab) to remove the bacterial plaque on the tongue dorsum by scraping it from dorsal to ventral for about 60 seconds. The individuals belonging to the wine tasters group, were asked to perform a sampling before and after a wine tasting (composing the groups prov1 and prov2 respectively). Some individuals from both control and wine taster groups were also asked to collect a nasal sample by introducing a new swap inside the nostril and rotate the swab several times against the nasal wall. Samples were stored in a sterile tube filled with RNAlater (Thermo Fisher Scientific, Waltham, MA, USA). All the samples were stored at -20ºC) until DNA extraction.

3.3. DNA Extraction

DNA was extracted with the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The concentration of the isolated DNA was quantified with a Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific). All samples were stored at -20ºC.

3.4. 16*S rRNA* Amplification, Library Preparation, and Sequencing

A two-step Polymerase Chain Reaction (PCR) approach was used to amplify the V3-V4 hypervariable regions and index an individual sample prior to pooling and sequencing. First, the target regions were amplified using universal primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTACHVGGGTATCTAATCC-3') (İriboz *et al.*, 2018) in a PCR mix including 5 µl of DNA template at a concentration of 10 ng/µl, 5 U of Taq PCR Master Mix Kit (Qiagen), 0.4 µl of each primer and 3.2 of distilled water, leading to a total reaction volume of 14 µl per sample. Thermocycling conditions consisted of an initial denaturation at 95ºC for 15 min followed by 40 cycles at 95ºC for 30 s, annealing at 55ºC for 1 min, and elongation at 72ºC for 30 s. A final elongation at 60ºC for 5 min was also adopted. Electrophoresis of the PCR products were used to check for amplicon quality and size by running 2 µl of PCR product and 1 µl bromophenol blue on a 2% agarose gel stained with Biotium GelRed Nucleic Acid Gel and visualized through a digital imaging system.

Subsequently, an indexing PCR based on a dual barcoding strategy was run using a combination of i5 and i7 indices (7 bp each). The reaction contained 7 µl of 2x Kapa HiFi Hot Start, 0.7 µl of each index, 2.8 µl of distilled water and 2.8 µl of DNA, in a total reaction volume of 14 µl per sample. Thermocycling conditions were an initial denaturation at 95ºC for 3 min, followed by 10 cycles at 95ºC for 30s, annealing at 55ºC for 30 s, and elongation at 72ºC for 30 s. The final elongation was at 72ºC for 5 min. The confirmation of index incorporation in each sample was performed on a 2% agarose gel, expecting an amplicon size of approximately 650 bp. A PCR clean-up using AMPure XP beads (Beckman Coulter, Brea, CA, USA) with a ratio of 0.8 µl of magnetic beads to 1 µl of PCR product was used to remove primer-dimers, reagent leftovers, and unspecific amplification products. The purified DNA was resuspended in an elution buffer. The success of this procedure was tested with 2% agarose gel stained with Biotium GelRed Nucleic Acid Gel Stain, expecting a band corresponding to the amplicon size of 460 bp. Finally, individual libraries were quantified using a BioteK™ Epoch™ Microplate Spectrophotometer (Thermo Fisher Scientific) and pooled. Lastly, the library was

validated through quantitative PCR. Precise sizing and quantification of the pool were obtained by using a TapeStation 2200 (Agilent Technologies, Santa Clara, CA, USA). The pool was sent to NovoGene (Cambridge, UK) and sequenced on an Illumina MiSeq sequencer with a 2x150bp paired-end configuration.

3.5. Sequence processing and alignment

The raw sequence data was provided by Novogene, 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 2-2021.4. (Bolyen *et al.*, 2019) The imported sequences were analyzed passing through filtering, denoising, dereplication, chimera identification and merged through DADA2 (Callahan *et al.*, 2016), a quality control package in QIIME 2, with the default parameters. The quality control will correct amplicon sequence errors and produce an output feature table with high-resolution amplicon sequence variants (ASVs), which registers the quantity of each ASV in each sample. Before these new methods, such as the ASVs, the quality control was performed by clustering the quality scores into OTUs based sequences, on a fixed dissimilarity threshold, typically 3% (Estaki *et al.*, 2020). ASVs method it is possible to distinguish sequences differing by only one nucleotide, not having a fixed threshold predicting that this will increase the taxonomic resolution (Callahan *et al.*, 2017).

Although, after denoising methods, the features given from our data have the highest resolution, it is important to know the taxonomic affiliation of the microbes. QIIME2 offers a plugin to predict the most likely taxonomic affiliation, we used the Naive Bayes classifier trained on the Greengenes v13_8 (DeSantis *et al.*, 2006), SILVA 138 (Quast *et al.*, 2013), RDP18 (Maidak *et al.*, 1997) and HOMD (Human Oral Microbiome Database)(Chen *et al.*, 2010) 99% OTUs databased, coincident with the microbial genomes databased used in the alignment reads. After taxonomic assignment, it was performed the removal of mitochondrial and chloroplast ASVs.

3.6. Statistical Analyses

To illustrate the taxonomic abundance in each sample for each taxonomic level was built a bar plot. The differences between the control and wine tasters' groups (prov1 and prov2) were tested using the Mann-Whitney U test in SPSS v.27, comparing the relative frequencies calculated for each ASV. Lastly, heatmap and a hierarchical clustering were constructed using the R package *phyloseq* (Fukuyama, 2020).

The alpha-diversity evaluates the microbiome diversity, which combines the richness (number of distinguishable) and evenness (taxa distribution) within a sample. The beta-diversity indicates the differences in taxa structure between samples. Two metrics of alpha diversity, namely the ASVs abundance and Shannon diversity index were computed (Shannon, 1948). Beta-diversity metrics were calculated through Bray-Curtis dissimilarity (Bray & Curtis, 1957). For the diversity analyses, rarefaction curves were performed to examine which sampling depth to use.

The comparison of alpha and beta diversities between the control and wine tasters' groups (prov1 and prov2) was performed by using the nonparametric Kruskal-Wallis H test and PERMANOVA with 999 permutations respectively, in QIIME2. PERMANOVA is a statistical test that evaluates if the samples from the same group are more similar to each other than to the samples from another group. To understand if there was potential clustering of the samples analyzed, it was performed a principal coordinated analysis (PCoA) through EMPeror (Vázquez-Baeza *et al.*, 2013), using the Bray-Curtis matrix.

The variables analyzed by the administrated questionnaire were also compared with the data obtained from the QIIME analysis. First, concerning the dietary habits of both wine tasters and the control group (59 individuals) different foods were classified according to their taste (bitter, sweet, sour, and astringent) and the frequency of consumption. For comparison of variables, was considered the mean frequency of each individuum per taste and converted into five categories (Category 1 – 0-20%, Category 2 – 20-40%, Category 3 – 40-60%, Category 4 – 60-80% and Category 5 – 80-100%) for a better understanding of data distribution. A descriptive analysis was performed for this dataset. To identify if there were significant differences between the two groups was carried out a Crosstab with a Chi-Square T-test. The same tests were also applied for the consumption of condiments.

Regarding, oral hygiene, smoking habits, and alcohol consumption it was accomplished a descriptive analysis and Crosstab with a Chi-Square T-test, to investigate significant differences between the two groups, and associations between the variables and be part of the wine taster or the control group. Within the wine tasters' group, to identify if there were any association between different durations of career and the frequency of tastings, the number of tastings in the last 15 days and the most frequent tasted wine was accomplished a Spearman correlation.

Finally, a Spearman correlation was also used to investigate if existed associations between dietary habits and oral hygiene habits, with the observed taxa. All tests were performed with a 5% level of significance.

4.RESULTS

4.1 Sequencing data and taxonomy assignment

Samples from the tongue of 59 individuals were collected, 30 belonging to the control group and 29 to the wine taster group. For the wine taster group two samples were collected, before and after wine tasting. One sample from the wine taster group was excluded because there was not enough product for amplification, making a total of 87 samples. The samples collected from the nostril were collected from five individuals of the wine taster group and six of the control group The raw reads obtain were a total of 44,728,156. The minimum number of reads per sample was 6516 and the maximum was 1,044,745 reads. With a mean of 451,799.5 reads per sample. After quality filtering, 41,428,645 reads remained. Due to the two sample types, nasal and tongue dorsum, the data were analyzed separately.

4.2. The Tongue Dorsum Microbiome

Regarding the tongue samples, the raw reads obtain were a total of 40,776,809. The minimum number of reads per sample was 6516 and the maximum was 1,044,745 reads. With a mean of 468,699 reads per sample. After quality filtering, 37,618,426 reads remained. The control group (495,738) showed a higher average number of reads per individual than the wine taster group (399,057) (Figure 3).

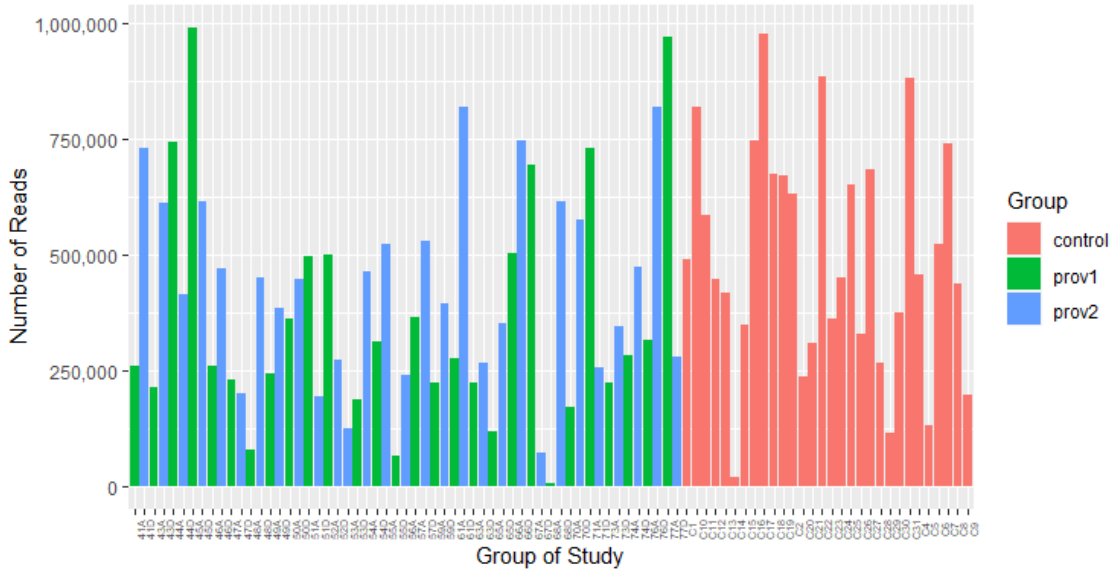

Figure 3. Bar chart representing the number of reads per individual and per group tongue swab samples) – prov1 in green (collected sample before wine tasting); prov2 in blue (collected sample after wine tasting) and control samples in red.

The reads with higher quality were assigned to features, a type of feature is ASVs, which are amplicon sequence variants that differ by only one nucleotide. Samples assigned to zero features were removed, remaining 80 samples. Regarding the 80 oral samples,

there were identified in total 837 ASVs of a total frequency of 375,930 reads. The mean frequency of ASVs per sample was 4,699.125 (range:4,0 – 38,394.0). After sample rarefaction, 15,900 reads (4.23%) and 53 samples (66.25%) were retained. 20 samples were preserved from the control group, and 33 from the wine taster group. This group is divided into samples collected before (Prov1) and after (Prov2) a wine tasting, retaining 15 and 17 samples, respectively. The number of pairs retained were nine, 20 were lost, keeping only one of the two samples.

The alignment of the ASVs was performed with four different databases, Greengenes, SILVA, RDP, and HOMD.

Greengenes database: the ASVs were assigned to 55 taxa, and the OTUs identification at the genus level was possible for 20% of the ASVs, and 4.13% at the species level.

SILVA database: 51 OTUs were identified, with 19% of the ASVs identified up to the genus level and 2.7% at the species level.

RDP databased: 18 taxa were identified from which 1,8% of the taxa was assigned at the genus and species level.

HOMD database: 35 taxa were identified and identification at the genus level was possible for 9.2% of the ASVs, and 8.5% at the species level.

4.2.1. Comparison between groups

Greengenes database: the wine tasters group shared 31 out of 55 OTUs. The control group exhibited 17 OTUs that were absent in the wine tasters' group, and the wine tasters group showed 7 OTUs that were absent in the other group. Of these 7 OTUs only present in the winetaster groups, two were shared between prov1 and prov2, three only present in prov1 group and two only in the prov2 group.

SILVA database: the two groups shared 31 out of the 51 taxa. The control group showed 18 OTUs that were absent in the wine tasters' group, and the last group exhibited 8 OTUs that were absent in the control group. Of these 8 OTUs, two were shared between prov1 and prov2, four were only present in prov1 group and two only in the prov2 group.

RDP database: The two groups shared 11 out of the 18 taxa. The control group showed 4 taxa that were absent in the wine tasters' group, and this last group exhibited 3 taxa that were absent in the control group. Of these three taxa, one taxa was shared between prov1 and prov2 groups, and two were only present in the prov2 group.

***HOMD database*:** 35 OTUs were identified, from which 11 were shared by the two groups. The control group exhibited 14 OTUs that were absent in the wine tasters' group, and the last group showed 10 taxa that were absent in the control group. Of these 10 OTUs, two were shared between groups, two only present in the prov1 group and six only in the prov2 group.

4.2.2. Taxonomic classification of the taxa

Greengenes database: A large portion of the samples was failed to be identified (42%), and were labelled as Unassigned, 40% were only assigned as Bacteria. From the assigned taxa the dominant phylum was the Actinobacteria (10%), Firmicutes (3%), Proteobacteria (1%). The 18 OTUs that demonstrated higher relative frequencies are represented in Figure 4A.

SILVA database: 70% of the OTUs present in the samples were not possible to identify, 13% were identified as Bacteria. The dominant phyla were Actinobacteria (11%), Firmicutes (3%), Proteobacteria (2%). The 19 taxa that showed higher relative frequencies are illustrated in Figure 4B.

RDP database: Most of the taxa were identified as phylum Eukaryarchaeota (70%), Archea (22%), Bacteria (0,8%), Proteobacteria (0.7%), Actinobacteria (0.3%), Firmicutes (0.1%), and 4% of the taxa was not assigned to a specific taxon. The 14 taxa that demonstrated higher relative frequencies are showed in Figure 4C.

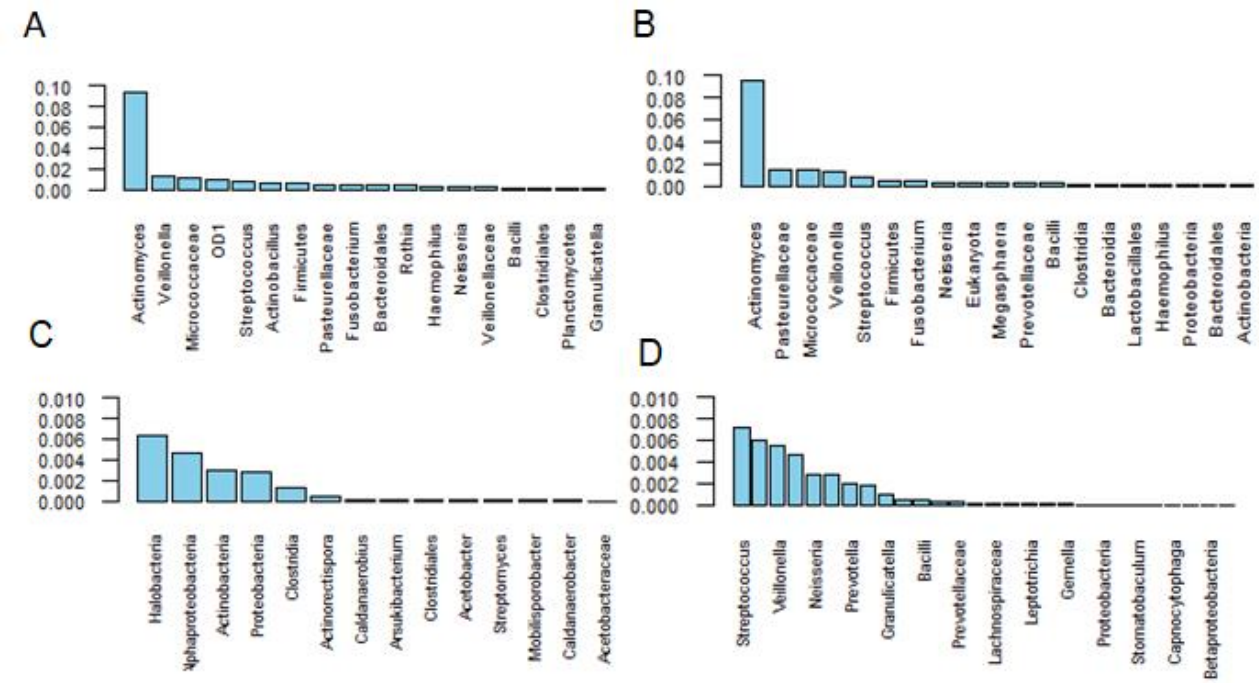

Figure 4. OTUs with the higher relative frequencies per reference database - (**A**) Greengenes, (**B**) SILVA, (**C**) RDP, (**D**) HOMD.

HOMD database: 96% of taxa present in the samples were Unassigned, and dominant phyla identified was Firmicutes (2%), Fusobacteria (0.5%) and Bacteroidetes (0.4%). The 14 OTUs showing the highest relative frequencies are represented in Figure 4D.

4.2.3. Distribution and abundance of the main taxa

Greengene database: By using this database it was possible to identify 9 phyla, 12 classes, 12 orders, 19 families, 22 genera, and 5 species (Figure 5). The mean number of taxa per individual was five. The most abundant OTUs were *Actinomyces* and *Veilonella* genera.

SILVA database: A total of 8 phyla, 10 classes, 19 orders, 22 families, 23 genera, and 10 species were identified. The mean number of taxa per individual was also five. The most abundant taxa were the Actinomyces and Pasteurellaceae (Figure 6). The genera *Actinomyces* and Pasteurellaceae were dominant.

HOMD database: A total of 5 phyla, 9 classes, 11 orders, 15 families, 20 genera, and 16 species were identified. The mean number of taxa per individual was two. The *Streptococcus* and Firmicutes were the most abundant OTUs (Figure 7).

RDP database: A total of 4 phyla, 5 classes, 6 orders, 6 families, 7 genera, and 7 species were identified. The mean number of OTUs per individual was three. The most abundant OTUs were from the Halobacteria and Alphaproteobacteria genera. RDP database performed worse than the other databases and was discarded for further analysis regarding oral microbiome composition.

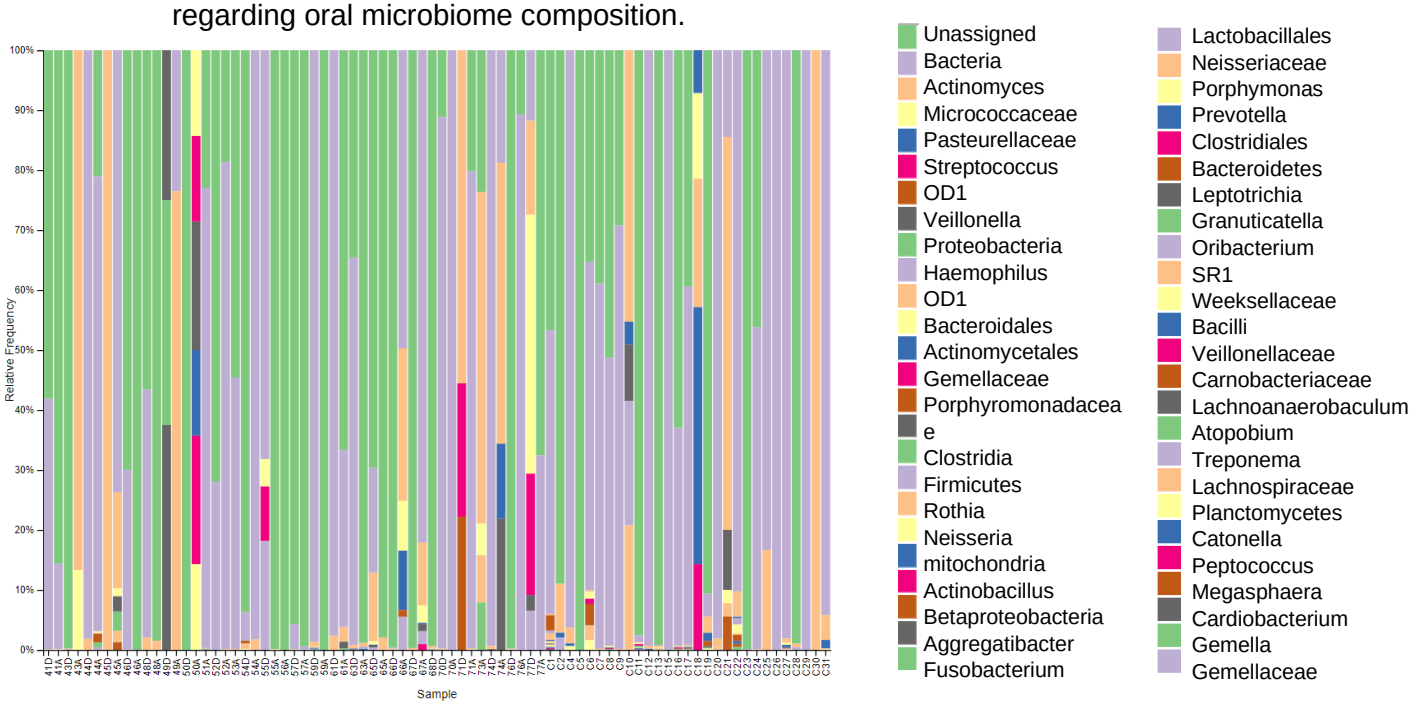

Figure 5. OTUs observed at each sample (up to the genus level) identified in Greengenes database. Sample names starting with C are from the control group, those ending in A are from the wine tasters' group that collected the sample before a wine tasting (prov1 group) and those ending in D represent wine tasters that collected the sample after a wine tasting (Prov2 group). The dominant colors in the chart represent the Unassigned taxa and the ones identified up to the dominant level (*Bacteria*).

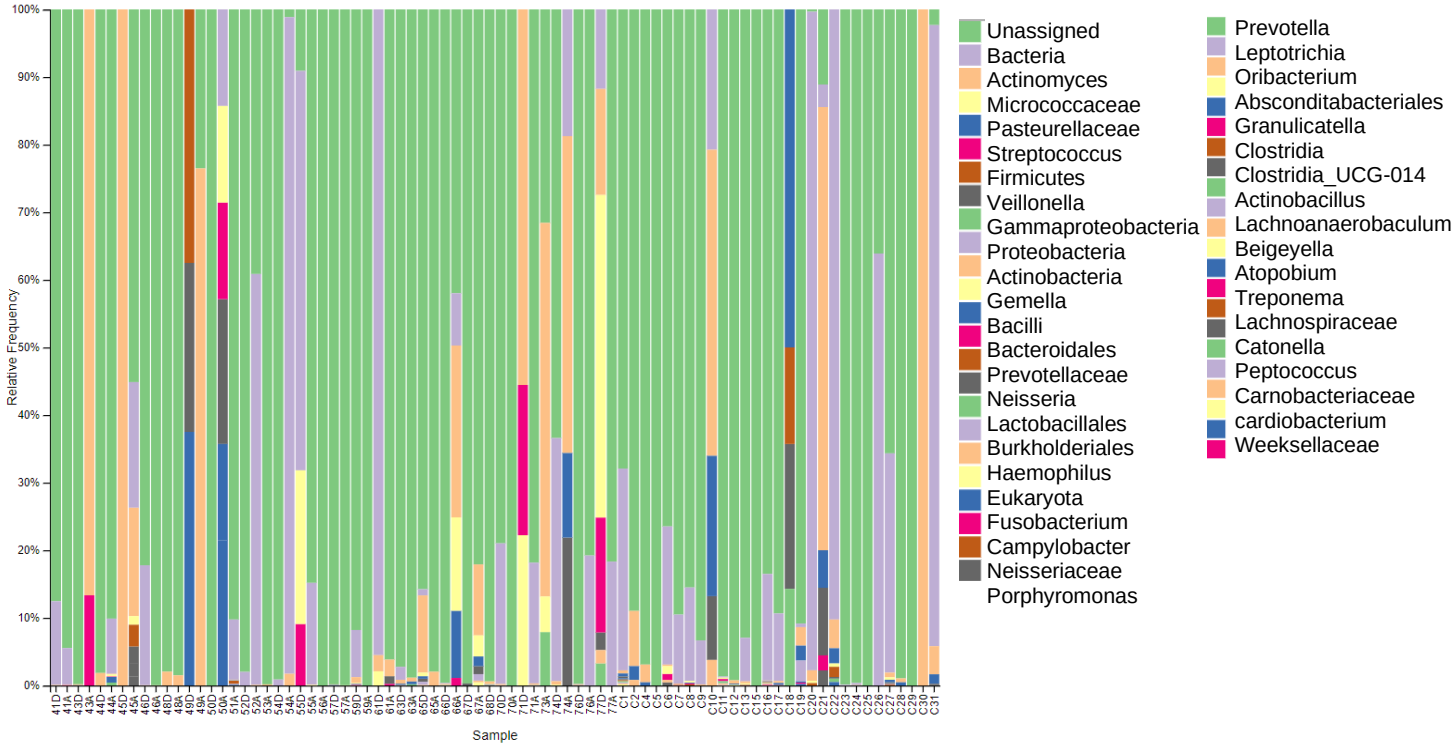

Figure 6. OTUs observed at each sample (up to the genus level) identified in SILVA database. Sample names starting with C are from the control group, those ending in A are from the wine tasters' group that collected the sample before a wine tasting (prov1 g group) and those ending in D represent wine tasters that collected the sample after a wine tasting (prov2 group). The dominant colors in the chart represent the Unassigned taxa and the ones identified up to the dominant level (Bacteria).

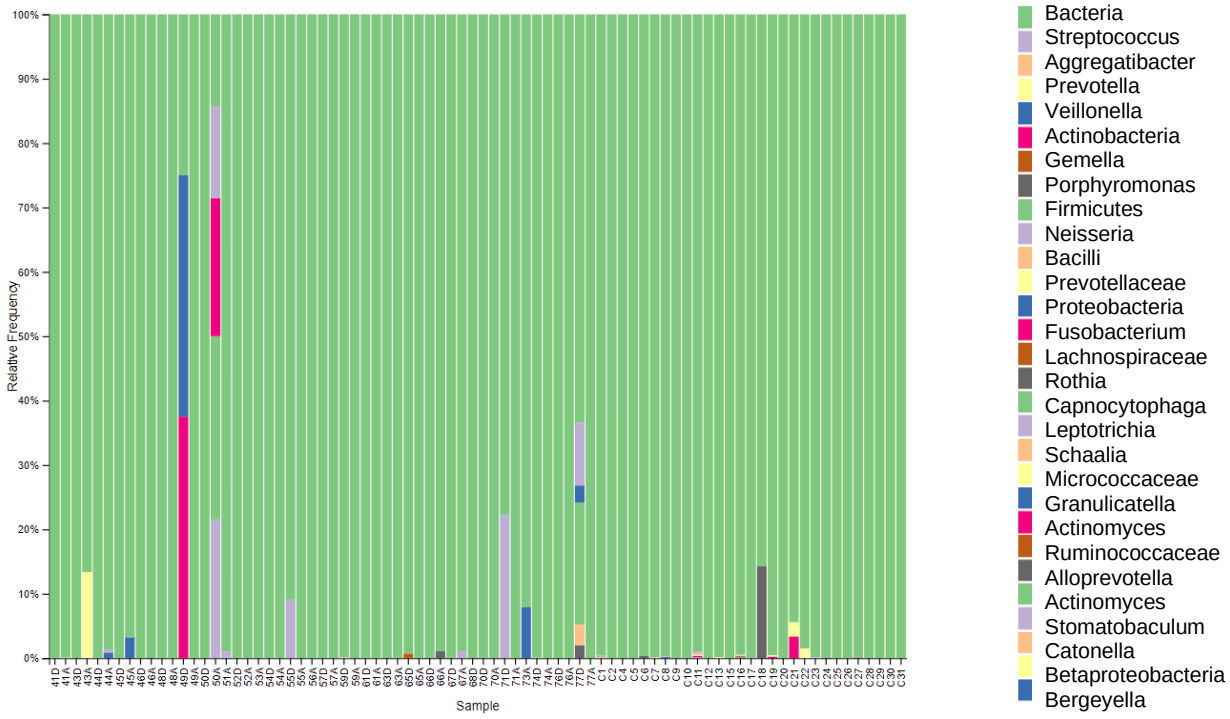

Figure 7. OTUs observed at each sample (up to the genus level) identified in HOMD database. Sample names starting with C are from the control group, those ending in A are from the wine tasters' group that collected the sample before a wine tasting (prov1 g group) and those ending in D represent wine tasters that collected the sample after a wine tasting (prov2 group). The dominant colors in the chart represent the Unassigned taxa and the ones identified up to the dominant level (Bacteria).

A heatmap for each database was built with taxa relative abundance and the top 50 taxa most abundant in the samples. As mentioned before, the database RDP was excluded as it was the less informative regarding the identification of taxa when comparing with the other databases. The ASVs identified as Unassigned, and *Bacteria* were excluded to achieve a clearer view of the identified taxa in the samples. Concerning the heatmap constructed with Greengenes database, the most abundant taxa is *Actinomyces* (Figure 9). We did not observe any OTU that was shared among all samples. There are two major clusters formed based on the relative frequency of *Actinomyces*. Both clusters are composed of with the 50 most abundant taxa individuals from the control and wine tasters' groups.

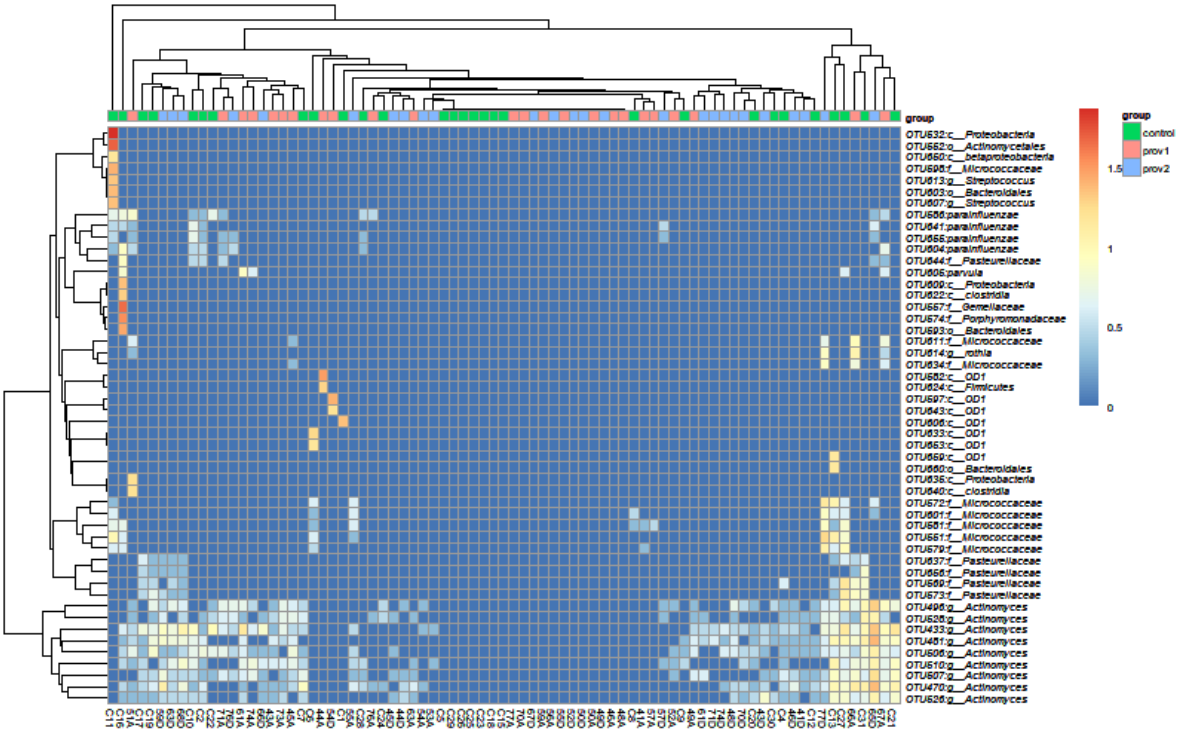

Figure 8. Top 50 most abundant OTUs up to the genus level per subject. Sample names starting with C are from the control group and those ending in D or A are from the wine taster group – Greengenes. Control group in green, prov1 in red and prov2 in blue

The heatmap constructed from the 50 most abundant OTUs identified by SILVA database, once again demonstrated that the OTUs with relative frequency between samples were the *Actinomyces* (Figure 9). There are two major clusters as in the heatmap presented before, based on the relative frequency of *Actinomyces*. Both clusters are composed by individuals from the control and wine taster groups.

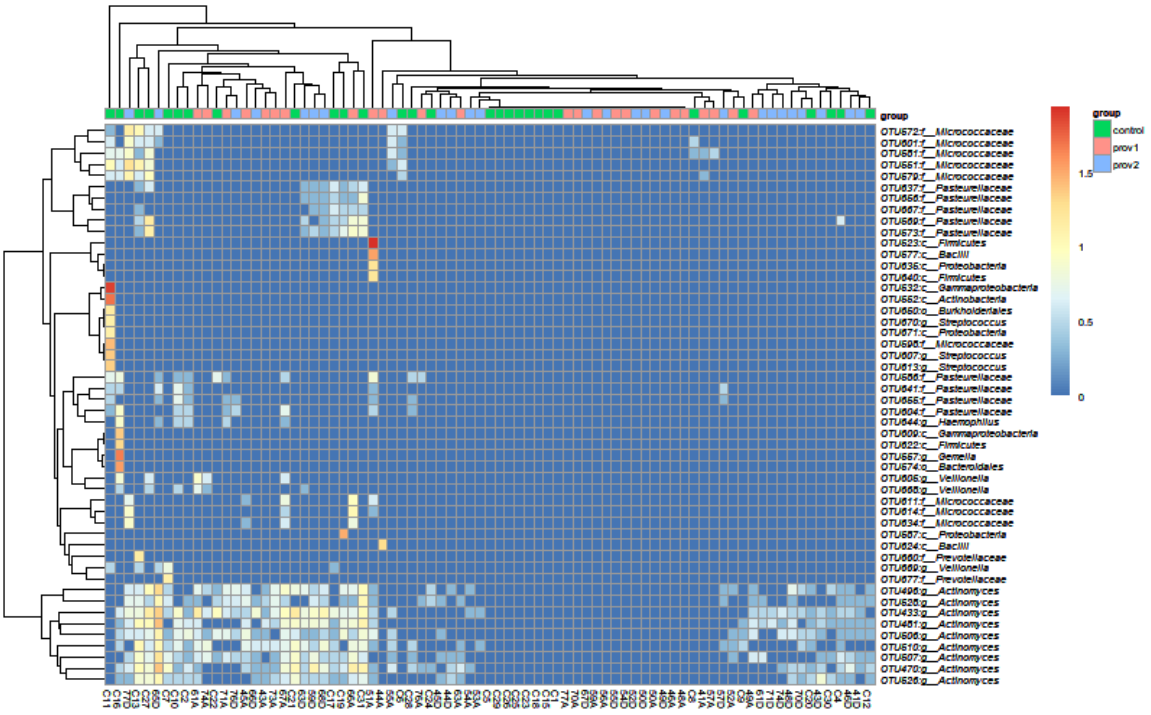

Figure 9. Top 50 most abundant OTUs up to the genus level per subject. Sample names starting with C are from the control group and those ending in D or A are from the wine taster group – SILVA. Control group in green, prov1 in red and prov2 in blue.

The *Aggregatibacter* genus showed the higher value of relative frequency of all the OTUs (Figure 10).

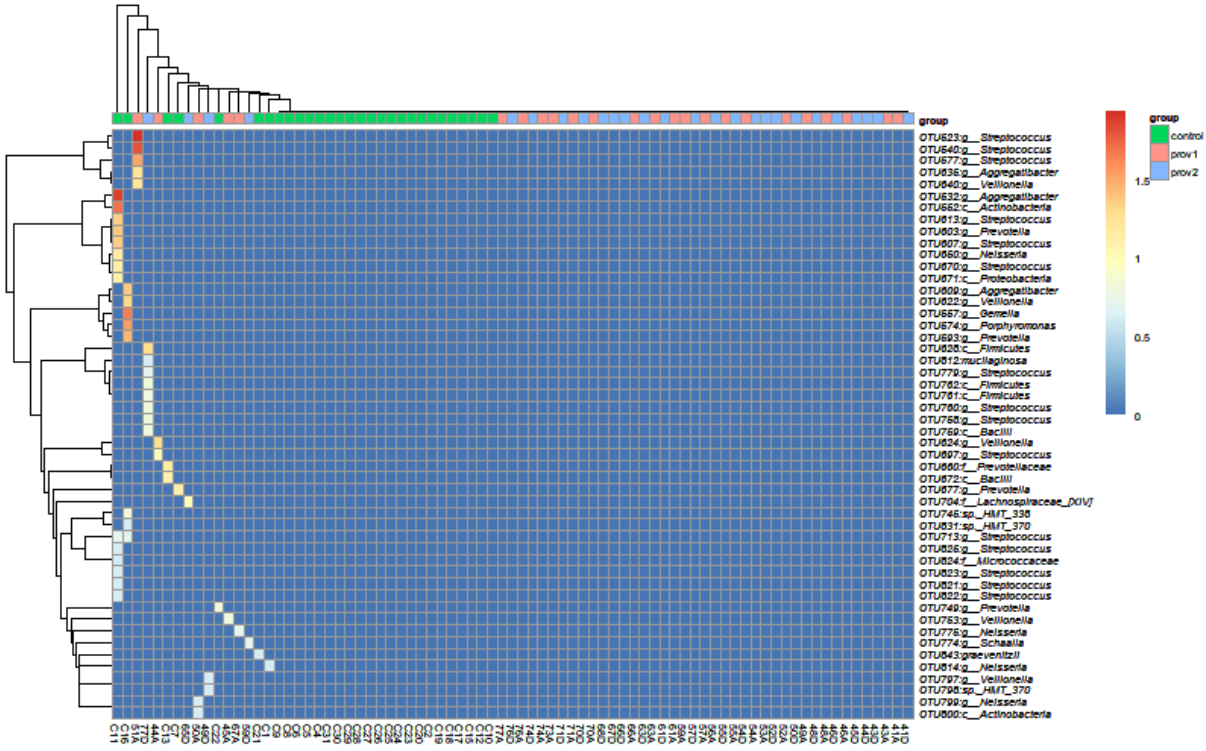

Figure 10. Top 50 most abundant OTUs up to the genus level per subject. Sample names starting with C are from the control group and those ending in D or A are from the wine taster group – HOMD. Control group in green, prov1 in red and prov2 in blue.

4.2.4. Microbiome differences between groups

To assess the differences in microbial taxonomy between the two groups, the bacterial composition was compared at class and genus levels using a Kruskal-Wallis Test. The taxa defined as Unassigned, and Bacteria were excluded.

4.2.4.1. Classes

Comparison between groups

Greengenes database: The Bacteroidia (p=0.013) and Betaproteobacteria (p=0.033) classes were significantly different between three groups - control group, prov1 (samples collected before wine tasting), and prov2 (samples collected after wine tasting). However, when the Bonferroni test is applied the only difference at the significant level remaining was the class Bacteroidia (0.012) between the control group and prov2.

HOMD database: the Bacteroidia (p=0.041) class was significantly different between the three groups, and when using Bonferroni correction, the difference at the significant level that remained was between the control group and prov2 the p-value slightly increased (0.044).

SILVA database: Gammaproteobacteria (p=0.015) and Bacteroidia (p=0.006) classes were significantly different between the three groups. Although when applying the Bonferroni test the significant level only remained between the control group and prov2 for the two classes (Gammaproteobacteria (p=0.030) and Bacteroidia (p=0.006)).

Relative abundance at the class level

Approximately 80% of the OTUs presented in the samples of all groups were Unassigned or only identified as Bacteria, not identifying phyla. Since this does not add relevant information to the study, this percentage of taxa was excluded. Therefore, to compare the composition of samples of the three groups, we use 20% of the total abundance.

***Greengenes database*:** From the ten most frequent classes of each group, the *Actinobacteria* is the dominant class in all groups, with the relative abundance of 14% in the prov2, 11% in the prov1, and 8% in the control group. The main differences are that the Bacilli had a higher relative abundance in the prov2 group (2%) than in the other groups (Figure 11). Clostridia had a higher abundance in the prov1 group (3%) than in the other two groups. Firmicutes had a higher abundance in the two groups representing wine tasters (prov1 – 0.15%; prov2 – 1.56%) than the control group (0.005%). On the

other hand, the control group (1.63%) showed a higher abundance of OD1 than the other two groups (prov1 – 0.34%; prov2 – 0.99%).

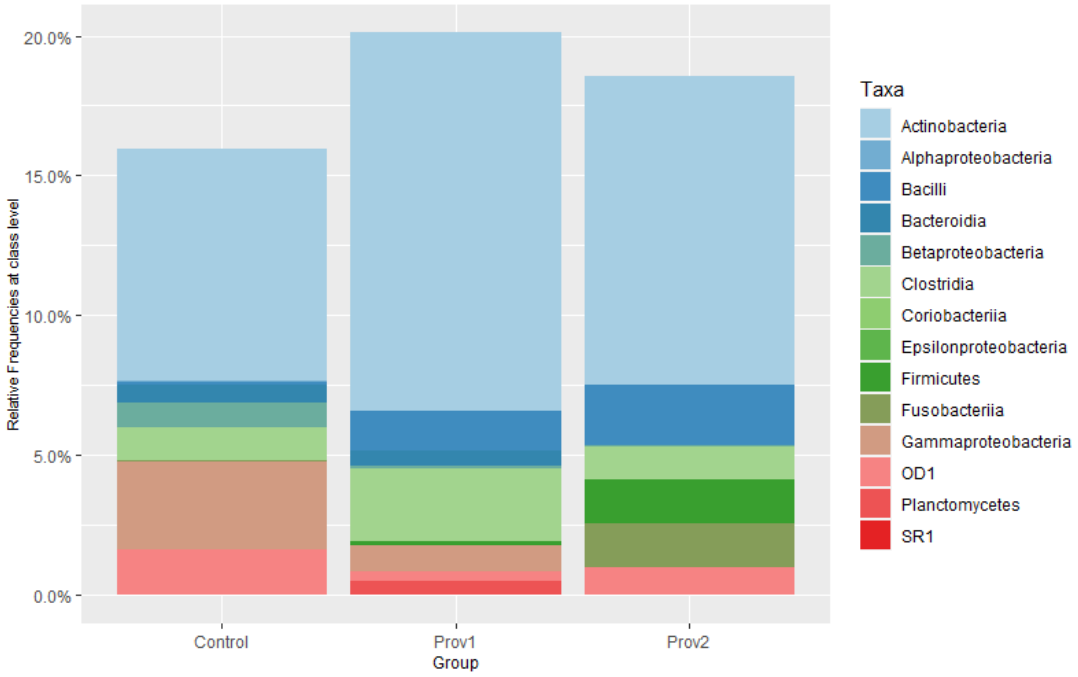

Figure 11. OTUs' relative abundance at the class level. The ten most abundant class of each group with Greengenes database.

***SILVA database*:** From the ten most frequent classes of each group, the Actinobacteria was the dominant class in all groups, with the relative abundance of 13.5% in the prov1, 11% in the prov2, and 8.3% in the control group (Figure 12). The main differences among groups were the higher abundance of Clostridia (0.01%), Bacteroidia (0.68%), and Gammaproteobacteria (4%) in the control group. Firmicutes *(*1.6%), Bacilli (2.2%), and *Fusobacterium* (1.6%) with greater abundance in the prov2 group than in the other groups, whereas Negativicutes (2.6%) were more abundant in the prov1 group than in the other two groups.

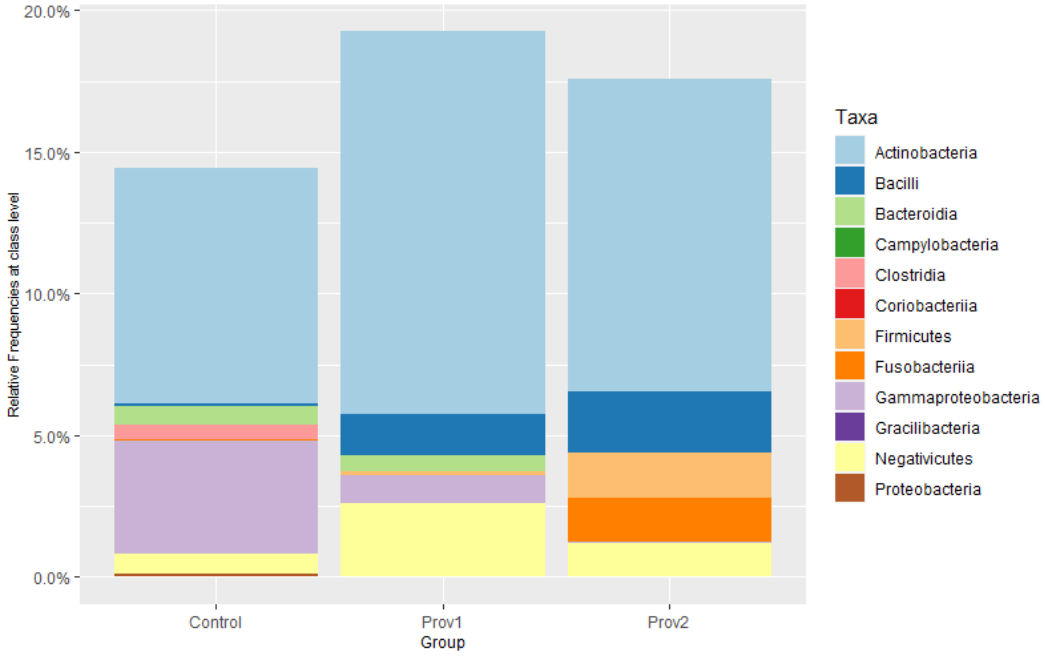

Figure 12. OTUs' relative abundance at class level. The ten most abundant taxa of each group identified in SILVA database.

HOMD database: The most dominant class among all groups is *Bacilli* with the relative abundance of 1.9% in the prov2 group, 0.9% in the prov1 group, and 0.02% in the control group. In this last group, the most dominant class was the Bacteroidia (0.64%), showing higher frequency than in the other two groups. The main differences are (1) the abundance of Actinobacteria (0.8%), Betaproteobacteria (0.8%) and *Firmicutes* (1%) in the prov1, and (2) Negativicutes (1.7%) and Fusobacteria (1.6%) had a higher abundance in the prov2 group.

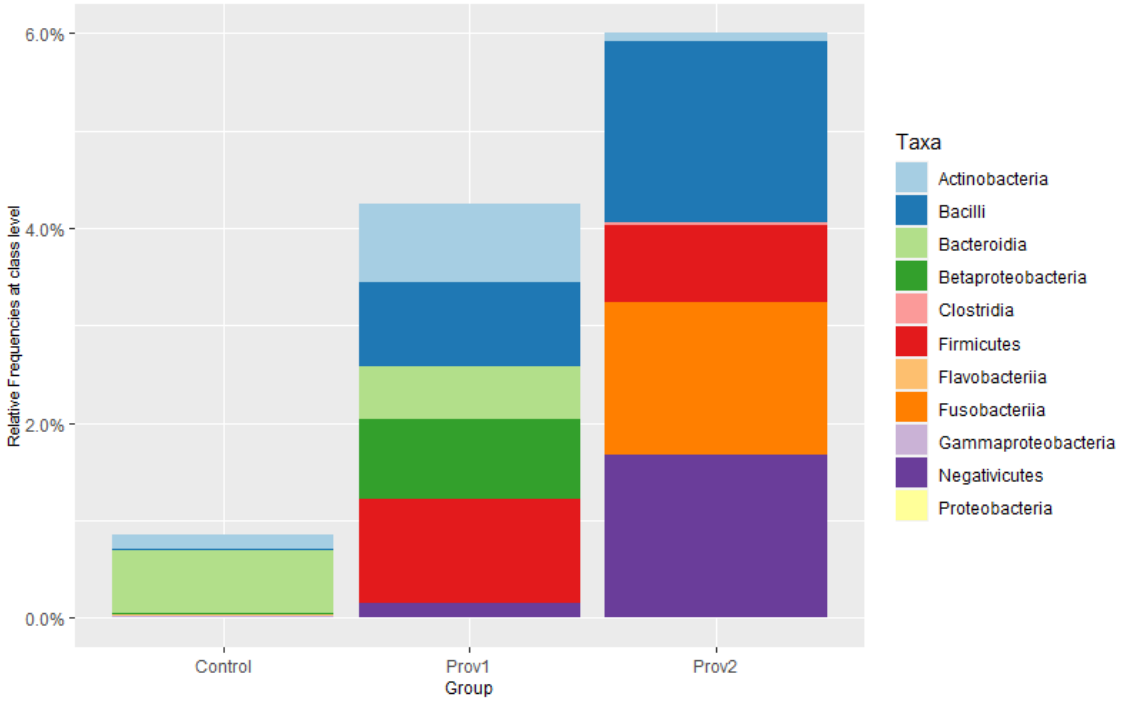

Figure 13. OTUs' relative abundance at class level. The ten most abundant taxa of each group identified in HOMD database

4.2.4.2. Genus

Comparison between groups

***Greengenes database*:** At the genus level, only about 20% of the total abundance in the three groups were identified as OTUs, excluding Unassigned and *Bacteria*. Some of the OTUs were only possible to be identified at the family level. *Neisseria* (p=0.028), *Fusobacterium* (p=0.019), *Bacteroidales* (p=0.017) and Pasteurellaceae (p=0.041) were significantly different among the three groups. However, when applying the Bonferroni test only remained Bacteroidales (p=0.018) significantly different between the control group and prov2, and *Fusobacterium* (p=0.022) between the control group and prov1.

HOMD *database*: the results from this database did not return significant differences in OTUs at the genus level between the three groups.

SILVA database: once again, some OTUs were only possible to identify up to the family level. Pasteurellaceae (p=0.006), Bacilli (p=0.049), *Neisseria* (p=0.028) and Prevotellaceae (p=0.026) were significantly different between the three groups. However, when applying the Bonferroni test the only significant differences that remained were Pasteurellaceae (p=0.018) between the control group and prov2, and between the control group and prov1 with the p = 0.021.

Relative abundance at the genus level

Greengenes database: The *Actinomyces* was the dominant genus among the three groups (prov1 – 12%; prov2 – 8%; control – 8%). The main differences were (1) a higher abundance of *Actinobacillus* (1.5%) and *Neisseria* (0.8%) in the control group than in the groups of wine tasters, (2) a higher abundance of *Streptococcus* in the wine tasters' groups (prov1 – 0.5%; prov2 – 2.15%) than in the control group, and (3) a higher abundance *Veillonella* (1.2%), *Rothia* (1%) and *Fusobacterium* (1%) in the prov2 group than in the other two groups (Figure 14).

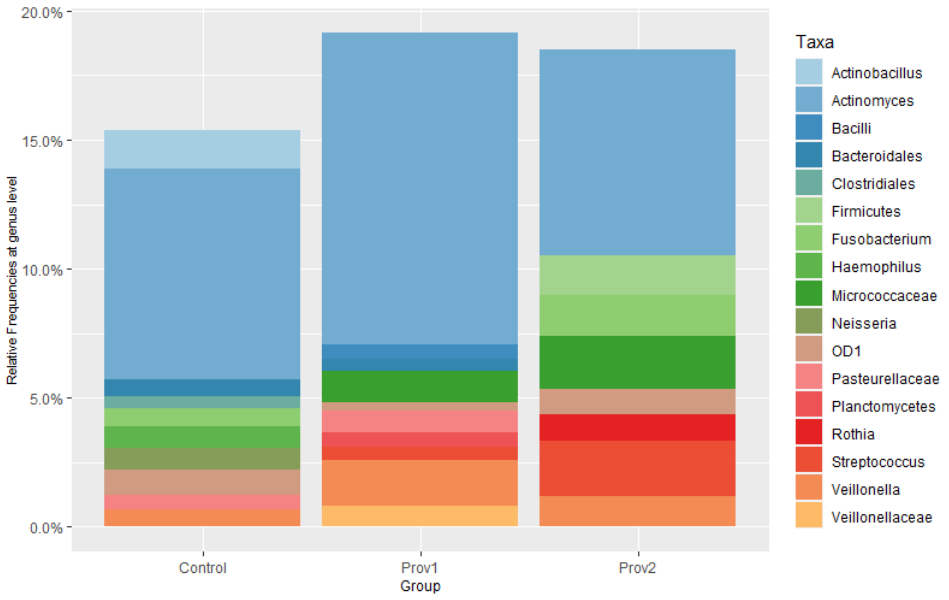

Figure 14. OTUs' Relative abundance at genus level. The ten most abundant OTUs by sample group with Greengenes database.

SILVA database: approximately 83% of the OTUs present in the samples of all groups was "Unassigned" or only identified as Bacteria. Therefore, to compare the composition of samples of the three groups, we use 17% of the total abundance. The dominant genus among the three groups was the *Actinomyces* in the same proportions as the Greengenes. Among the three groups the most evident differences are the higher abundance of Actinobacteria (0.08%), Micrococcaceae (3%) and *Streptococcus* (2%) in the prov2 group than in the other two groups. Pasteurellaceae (3%) more abundant in the control group than in the groups of wine tasters and *Veillonella* (1.8%) with higher abundance in the prov1 group (Figure 15).

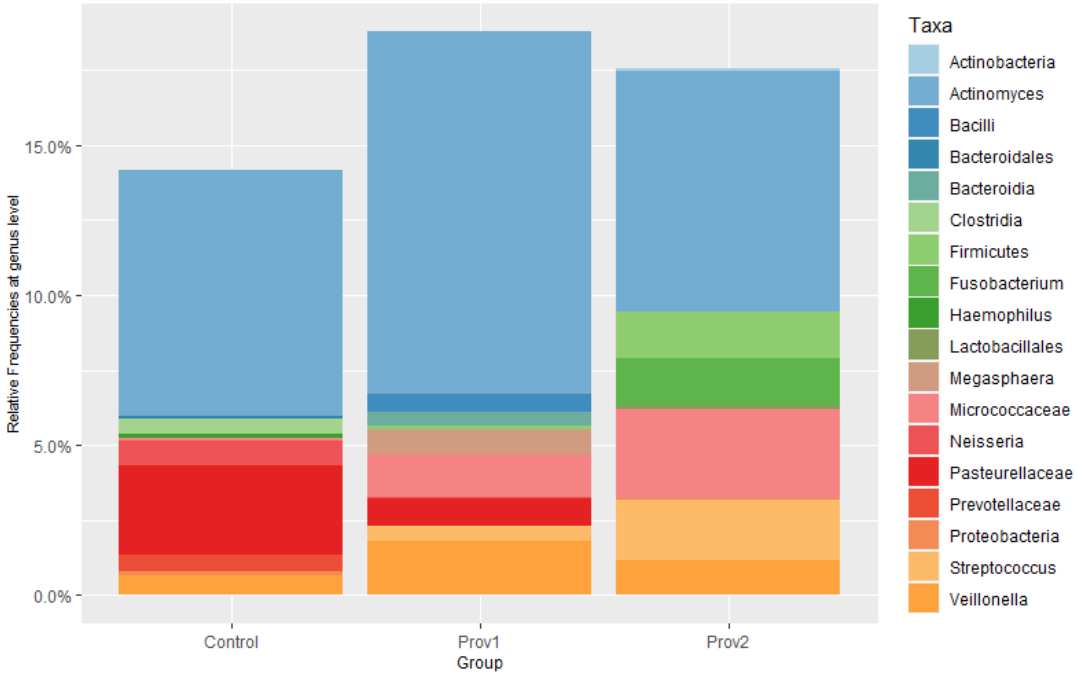

Figure 15. OTUs' relative abundance at genus level. The ten most abundant OTUs by sample group with SILVA database

HOMD database: Approximately 96% of the taxa present in the samples of all groups as Unassigned or only identified as Bacteria, Therefore, to compare the composition of samples of the three groups, we use the remaining 4% of the total abundance. In the control group was only possible to identify up to the class and genus level 1% of the taxa. The ten most frequent genus of each group are represented in the Figure 16. The main differences between groups are the higher abundance of *Alloprevotella* (0.5%), *Actinomyces* (0.1%) and Prevotellaceae (0.08%) in the control group. *Neisseria* (0.8%), Firmicutes (1%), Actinobacteria (0.8%) and *Prevotella* (0.5%) most abundant in the prov1 group than in the other two groups. And the higher abundance in the prov2 group of *Fusobacterium* (1.6%), *Rothia* (0.08%), *Streptococcus* (1.7%), *Veillonella* (1.7%) (Figure 16).

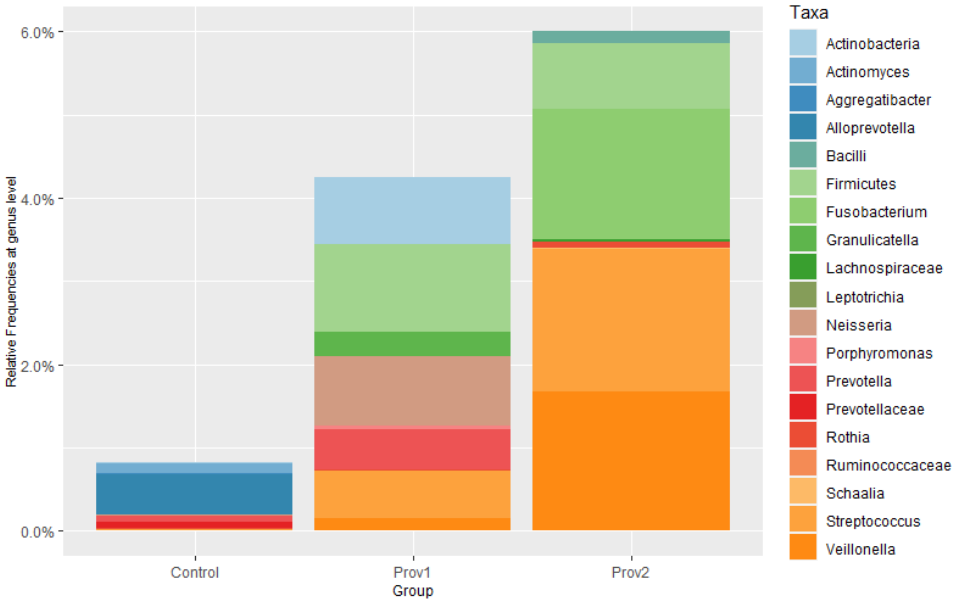

Figure 16. OTUs' relative abundance at *genus* level. The ten most abundant OTUs by sample group with HOMD database

4.2.5. Microbiome Diversity

The sampling depth was selected before the assessment of the diversity measures. Consequently, it was performed rarefaction curves for the Shannon index and ASVs abundance, per group. The rarefaction curves for the Shannon index showed that the plateau was reached over 300 sequence reads (Figure 17A). Regarding the rarefaction curves for the ASVs, the ones of wine tasters' groups progressively approached saturation, reflecting the species richness. The curve of the control group has the lower richness values for species because does not achieve a plateau (Figure 17B). The

samples were rarefied to 300 reads, which coincident with the plateau value, and keep the maximum number of samples and ASVs.

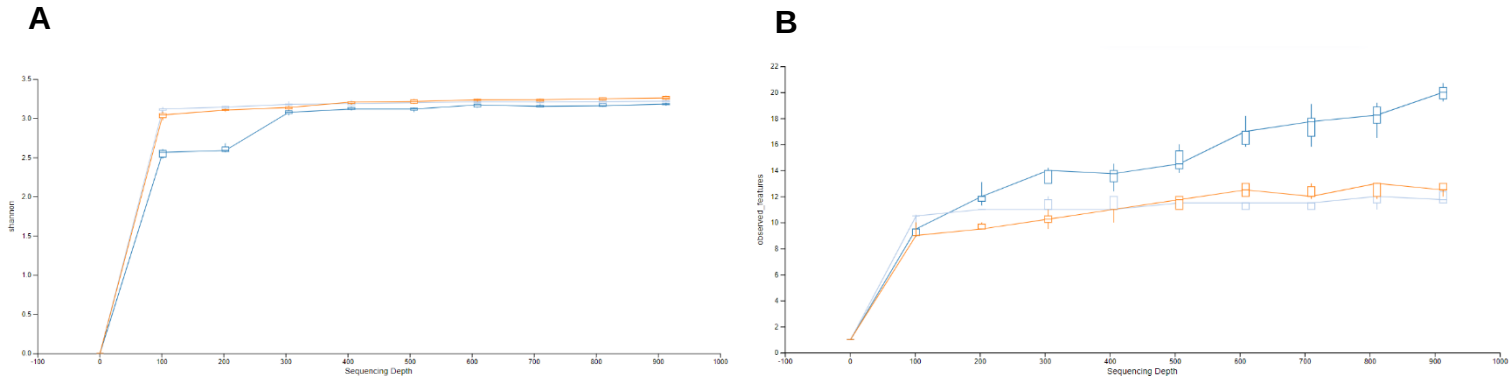

Figure 17. Rarefaction curves from (A) Shannon index and (B) the ASVs abundance per group. Control samples in dark blue, prov1 in light blue and prov2 in orange

4.2.5.1. *Alpha* Diversity

Shannon index and ASVs abundance are the measures used for the alpha diversity, which were compared between groups, and different categories of the following variables: gender, age, alcohol consumption, hygiene habits (tooth brushing and mouthwash use), frequency of visits to the dentist, frequency of consumption of food from the tastes sweet, bitter, sour, and astringent and wine taster career. Variables such as consumption of taste sweet, bitter, sour, and astringent were divided into 5 categories, according to the frequency of consumption (category 5 – very frequently and category 1 – never). Observing the Shannon index (Figure 18) and ASVs observation (Figure 19) it appears to exist a difference between the two groups of wine tasters and the control group, but it is not significant. Among the three groups the Shannon index values were not significantly different (p=0.91), nor for the ASVs abundance (p=0.53).

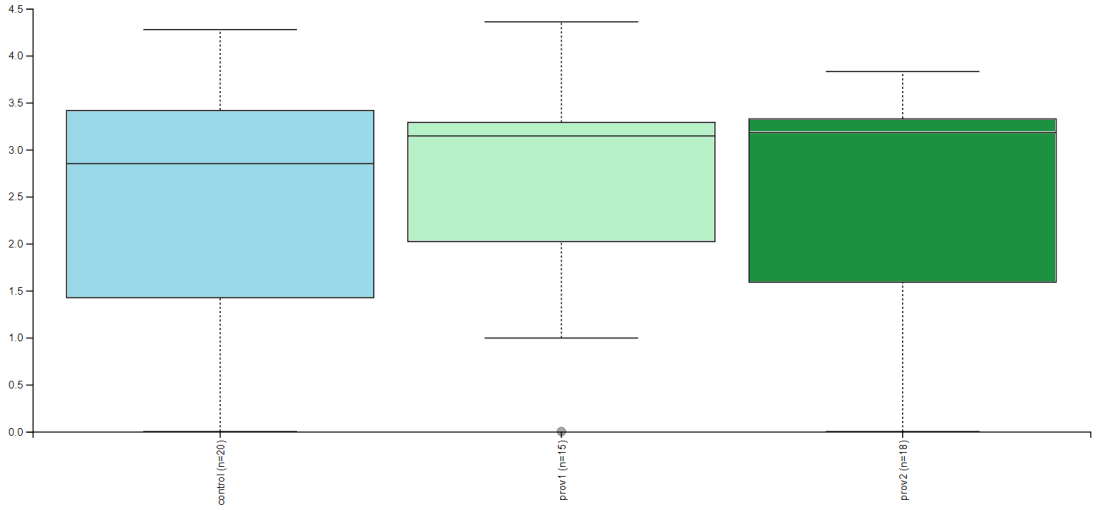

Figure 18. Boxplot chart depicting the distribution of the Shannon index in the three groups (mean: control (2.49), prov1(2.87), prov2(2.58)). Control samples in blue, prov1 in light green and prov2 in dark green.

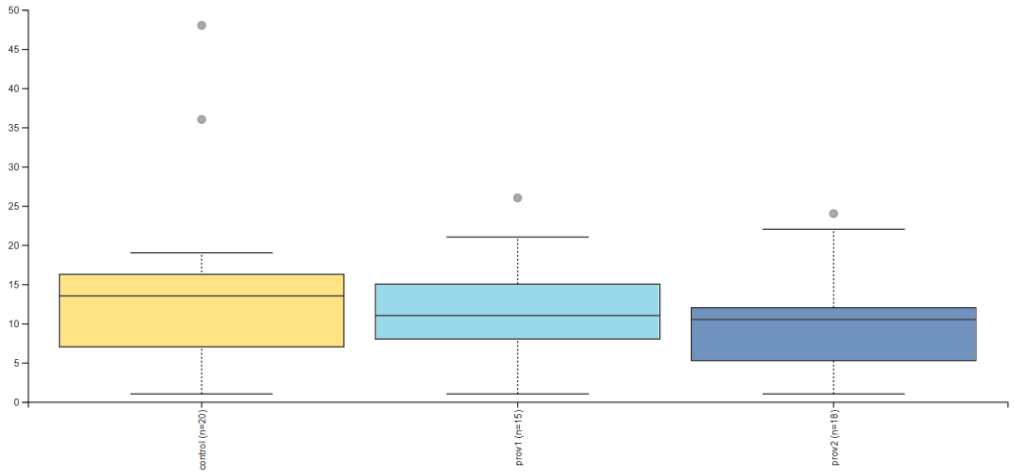

Figure 19. Boxplot chart depicting the distribution of the ASVs abundance in the three groups (mean: control (14), prov1(12), prov2(10)). Control samples in yellow, prov1 in light blue and prov2 in dark blue.

Regarding gender, the Shannon index and the ASVs abundance were calculated first for all the individuals and then distinctly according to the group. The Shannon indexes (Figure 20) for the two genders were practically the same, and not significantly different (p=0.86), for the gender according to the group (Figure 21) was also not significantly different (p=0.68).

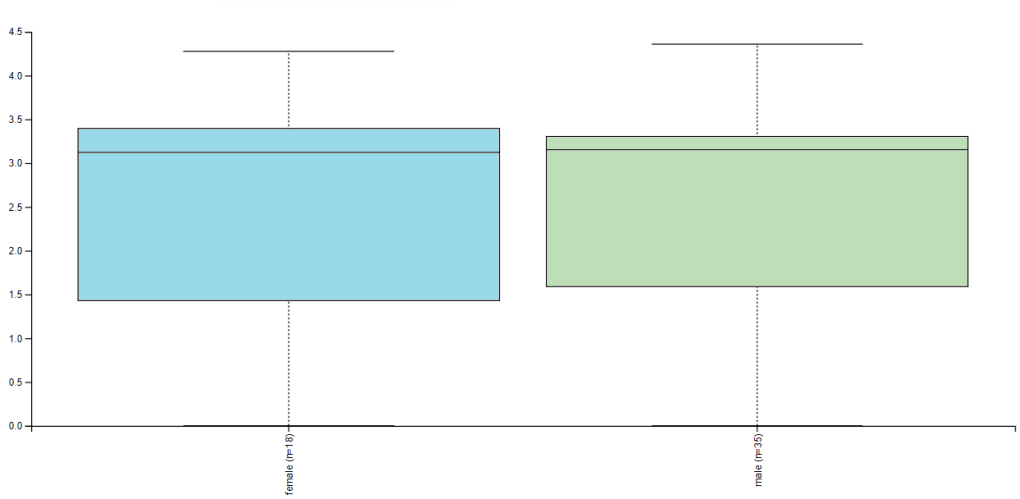

Figure 20. Boxplot chart depicting the distribution of the Shannon index in both genders (mean: 2.77 female (blue); 2.57 male (green)

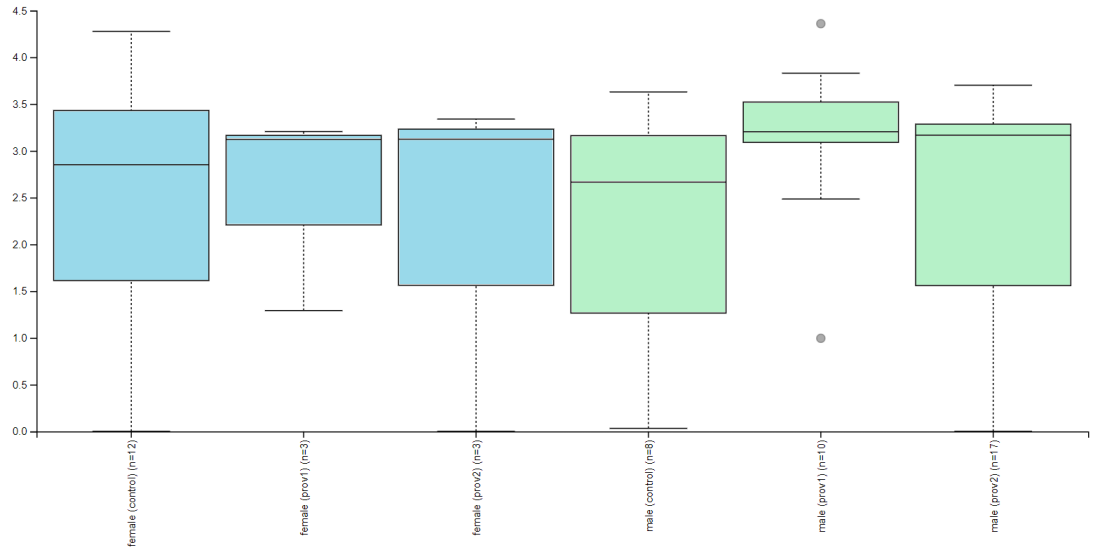

Figure 21. Boxplot charts depicting the distribution of the Shannon index in both genders according to the group (mean: female (control - 2.7, prov1 – 2.5, prov2 – 3.2); male (control – 2.1, prov1 – 3.1, prov2 – 2.2), female in blue and male in green

The comparison of the ASVs between genders (Figure 22) demonstrated lower counts in males, but it was not significantly different (p=0.20). Likewise, when dividing the gender according to the groups (p=0.27) (Figure 23).

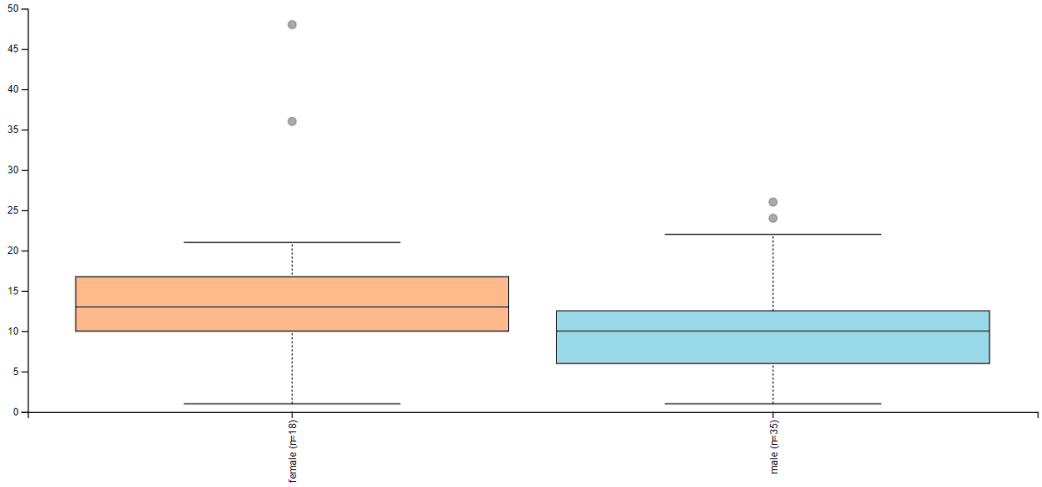

Figure 22. Boxplot charts depicting the distribution of the ASVs counts in both genders (mean: 14.77 (female); 10.63(male)). Female group in orange and male in blue

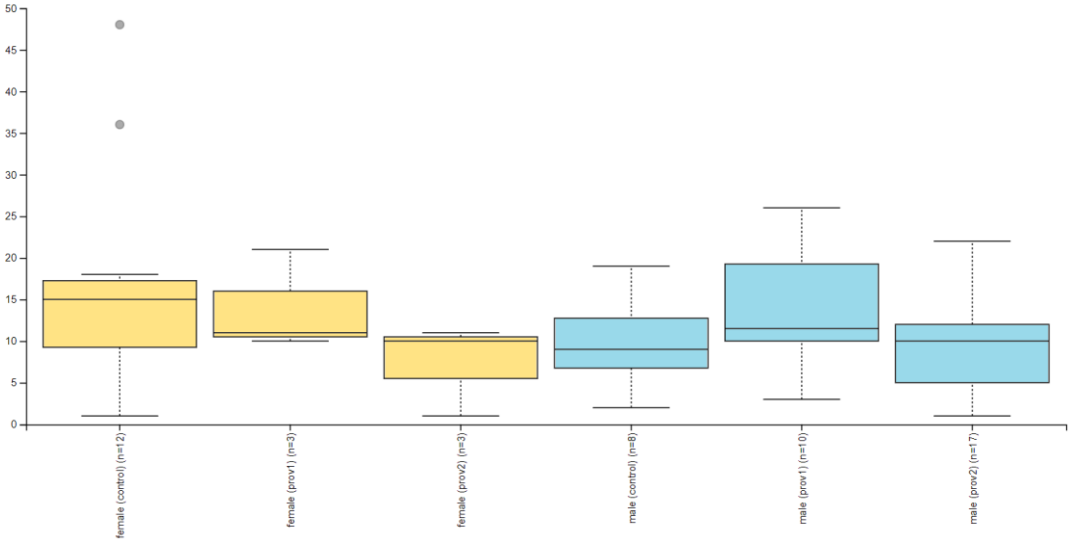

Figure 23. Boxplot charts depicting the distribution of the ASVs counts in both genders according the group (mean: female (control – 16.8, prov1 – 14, prov2 – 7.3); male (control – 9.8, prov1 – 13.7, prov2 – 9.2), female group in yellow and males in blue

The alpha diversity measures were also compared grouping the variables by categories of consumption of each taste, considering the control, the prov1, and the prov2 groups separated (Figure 24, 25, 26, 27). The categories were made based on the median percentage obtained from the frequency of consumption of the different foods referring to each taste (Category 1 representing the lower frequency of consumption – 0-20%, and Category 5 the higher – 80-100%). For each comparison, there was no significant difference between groups and categories. When the groups are separated by the

categories of bitter taste, category 3 of prov1 and prov2 groups obtained the higher and lowest Shannon index values, respectively (Figure 24). Comparing the ASVs count values the same result was obtained (Figure 25).

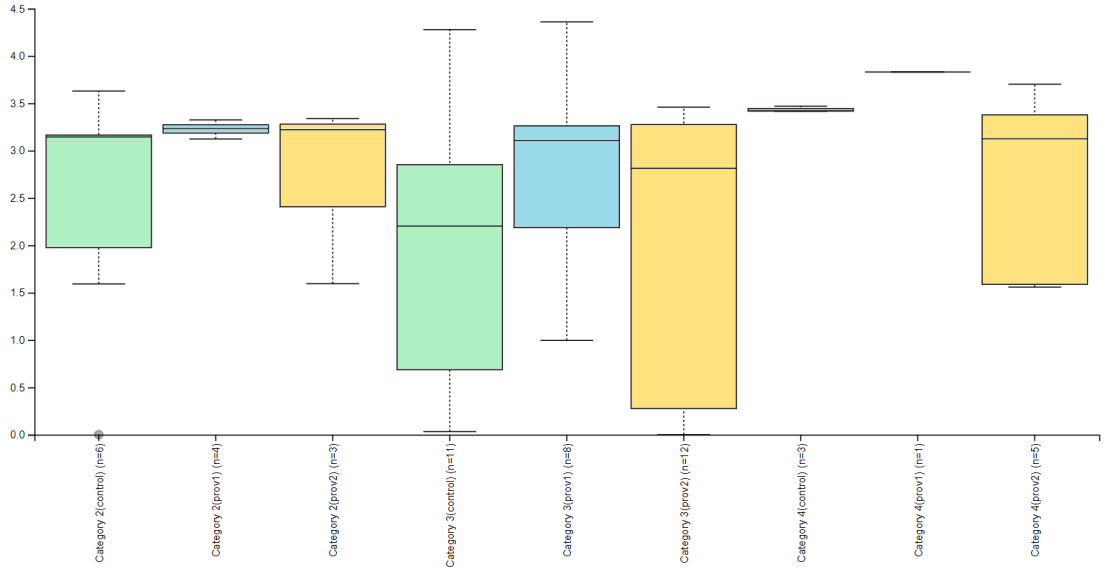

Figure 24. Boxplot charts depicting the distribution of the Shannon index in the different categories of bitter taste for each group (mean: control (cat.2 – 2.9, cat3 – 4.0, cat4 – 3.4); prov1 (cat2 – 3.2, cat3 – 2.8, cat4 – 3.8); prov2 (cat2 – 2.7, cat3 – 2.3, cat4 – 3.8); prov2 (cat2 – 2.7, cat3 – 2.3, cat4 – 2.7), p=0.30, control samples in green, prov 1 in blue and prov2 in yellow

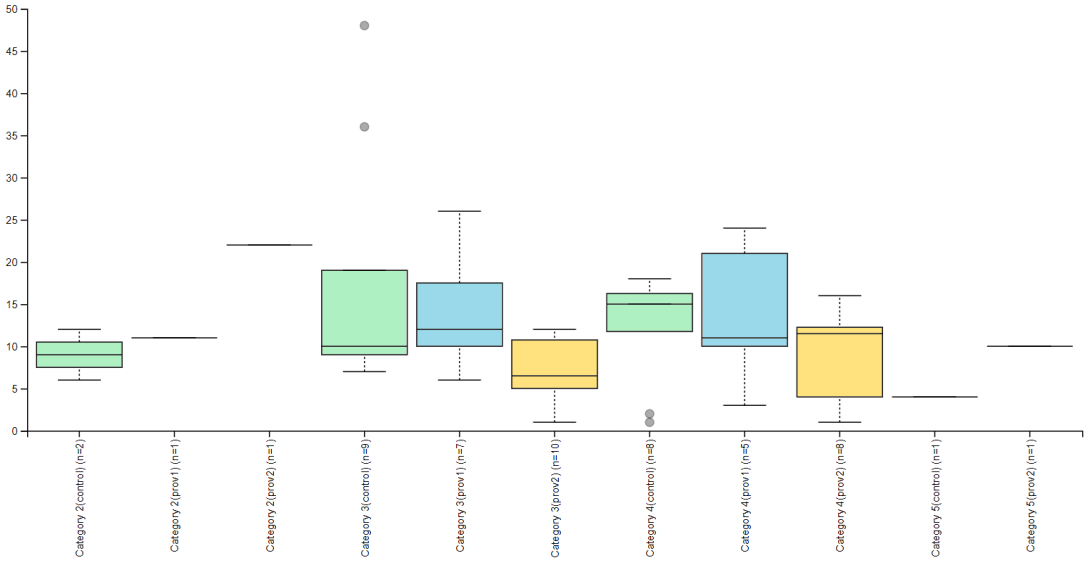

Figure 25. Boxplot charts depicting the distribution of the ASVs counts in the different categories of bitter taste for each group (mean: control (cat2 – 10.3, cat3 – 15.6, cat4 – 15.6); prov1 (cat2 – 11, cat3 – 13.8, cat4 – 24); prov2 (cat2 – 8.3, cat3 – 8.7, cat4 – 29), p=0.43, control samples in green, prov 1 in blue and prov2 in yellow

Regarding the variable “Higher frequency of consumption of sweet-related foods” separated by groups, category 3 of control obtained the highest and lowest Shannon index, category 3 of the prov2 group also obtained the lowest Shannon index value (Figure 26). Category 3 of the prov1 group obtained the highest ASV counts, and category 3 of control and prov2 groups obtained the lowest ASV count (Figure 27). The sour and astringent tastes are represented in Supplementary Figures 1 to 4.

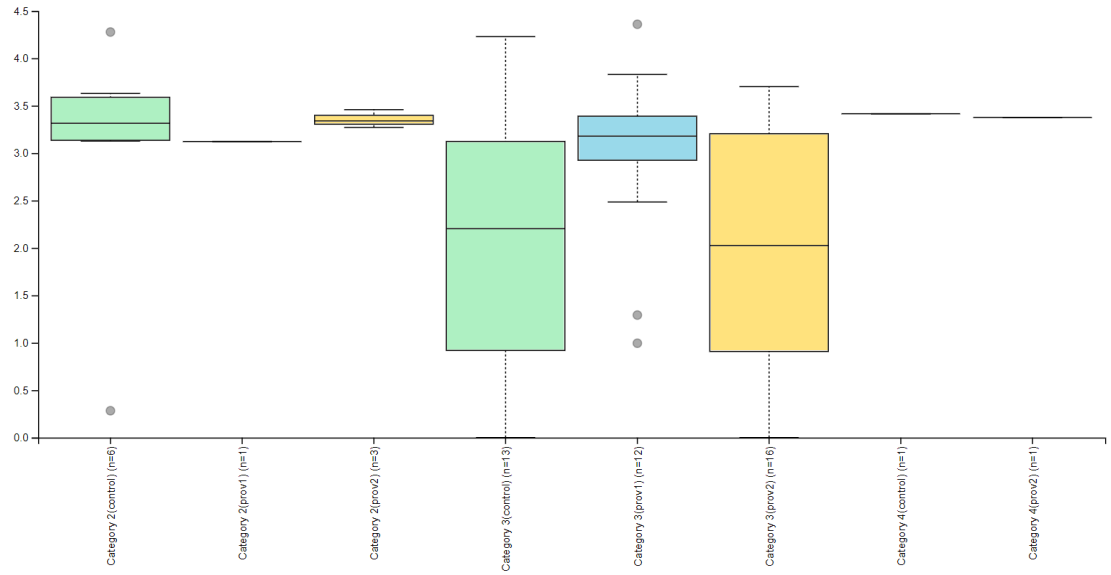

Figure 26. Boxplot charts depicting the distribution of the Shannon index in the different categories of sweet taste for each group (mean: control (cat2 – 2.99, cat3 – 2.2, cat4 – 3.4), prov1 (cat2 – 3.2, cat3 – 2.9); prov2 (cat2 – 3.6, cat3 – 2.3, cat4 – 3.4), p=0.12, control samples in green, prov 1 in blue and prov2 in yellow

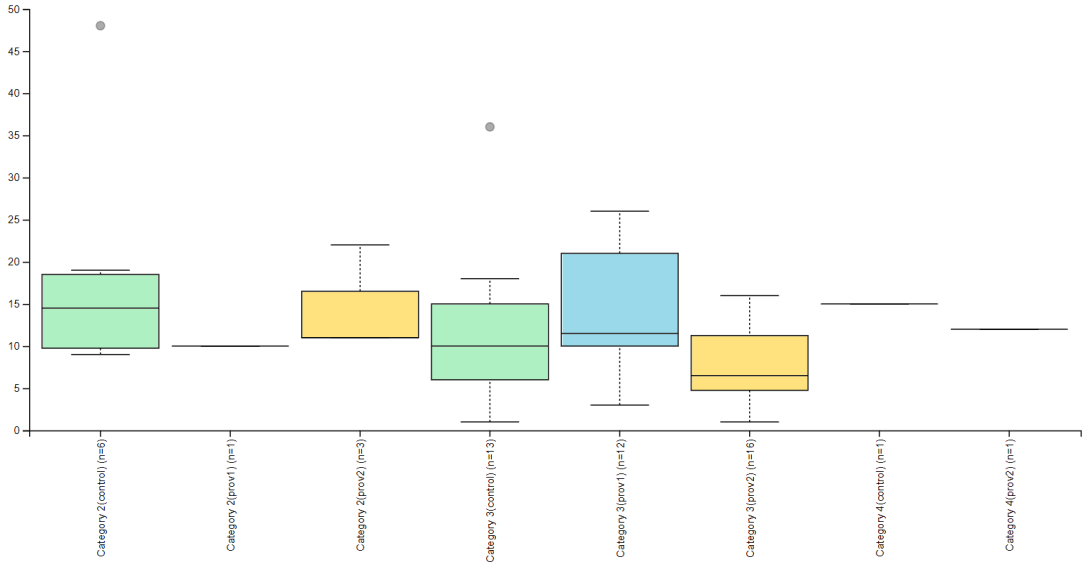

Figure 27. Boxplot charts depicting the distribution of the ASVs counts in the different categories of sweet taste for each group (mean: control (cat2 – 19, cat3 – 11.7, cat4 – 15); prov1 (cat2 – 10.5, cat3 – 14.4); prov2 (cat2 – 14,6, cat3 – 7.8, cat4 – 12), p=0.19, control samples in green, prov 1 in blue and prov2 in yellow

When comparing the Shannon index of the variable “Higher frequency of consumption of sweet-related foods” with all the samples together (Figure 28), there is a significantly difference in the richness of the samples between different frequencies of consumption (p= 0.04).

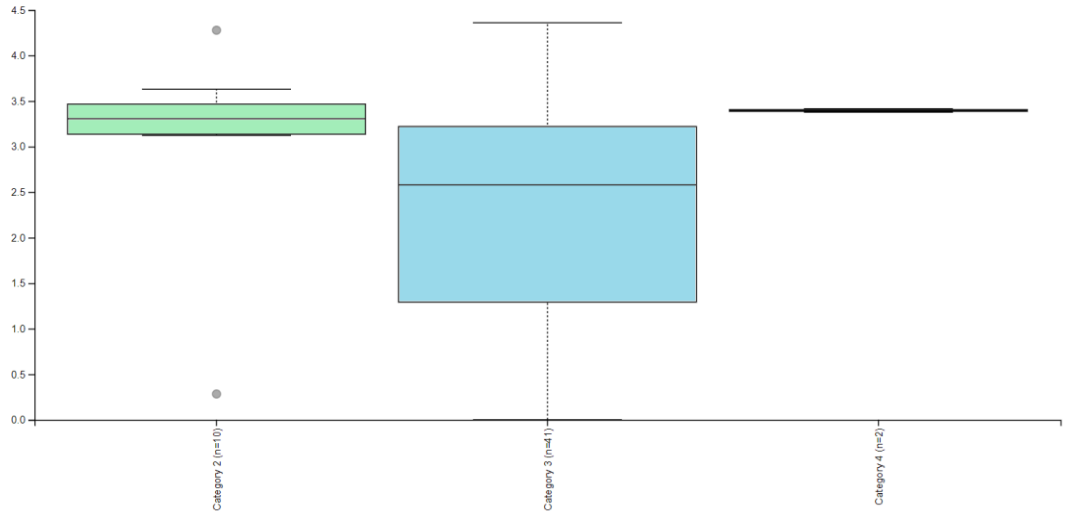

Figure 28. Boxplot charts depicting the distribution of the Shannon index in the different categories of sweet taste for all subjects (mean: cat2 – 3.1, cat3 – 2.5, cat4 – 3.4), category 2 in green, category 3 in blue and category 4 in dark blue.

Regarding the two groups of wine tasters, it was compared the alpha diversity according to the career length and frequency of attendance in wine tastings (Figure 29 and Figure 30). Comparing the Shannon index and ASV count with the different categories of wine taster career, 5 to 10 years of career from prov1 group showed higher value. The lowest Shannon index and ASV counts were obtained by category “10 to 15 years of career” from the prov2 group.

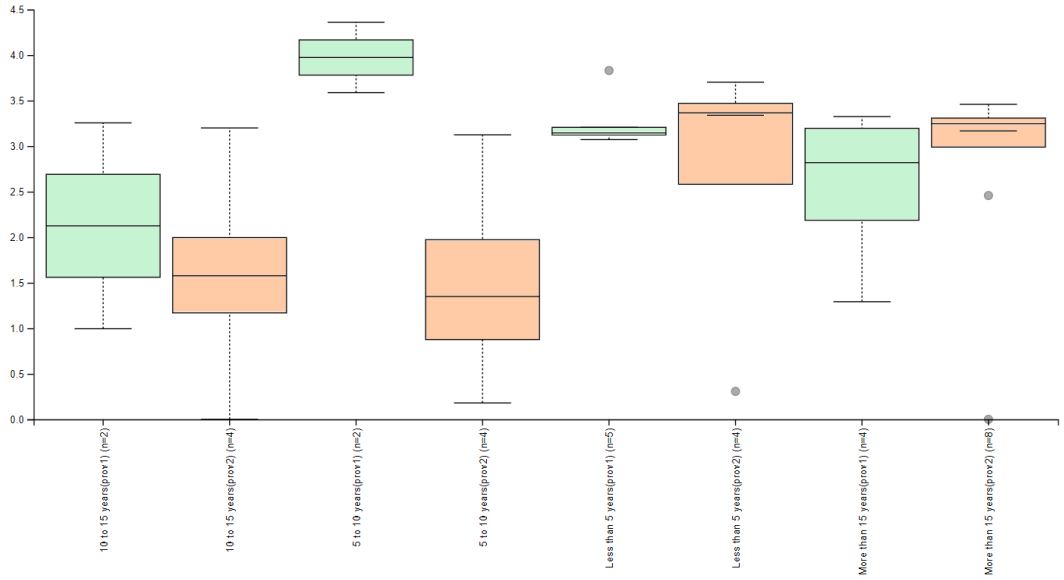

Figure 29. Boxplot charts depicting the distribution of the Shannon index in the different categories of career length of wine taster for each group (mean: less than 5 years (prov1= 3.3, prov2=2.7), 5 to 10 years (prov1 =3.9, prov2=1.5), 10 to 15 years (prov1 = 2.1, prov2 = 1.3), more than 15 years (prov1 = 2.6, prov2 = 2.9); p=0.09), prov1 samples in green and prov2 in red.

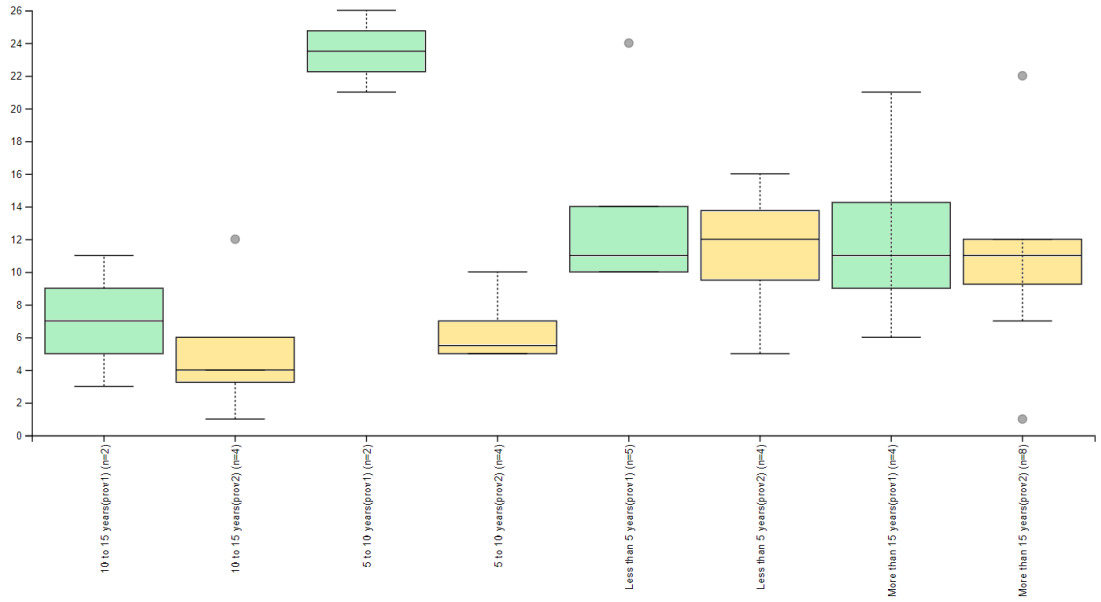

Figure 30. Boxplot charts depicting the distribution of the ASVs counts in the different categories of career length of wine taster for each group (mean: Less than 5 years (prov1 – 13.7, prov2 – 11.25); 5 to 10 years (prov1 – 23.5, prov2 – 5.3); 10 to 15 years (prov1 – 12, prov2 – 7); more than 15 years (prov1 – 12.3, prov2 – 10.8); p=0.08), prov1 samples in green and prov2 in orange.

When comparing the frequency of attendance in wine tastings between the two groups of wine tasters, with the Shannon index and ASV counts, the category of frequency “Less than one time per month”, obtained the highest index, and category “2 to 4 times per week”, the lowest value (Figures 31 and 32).

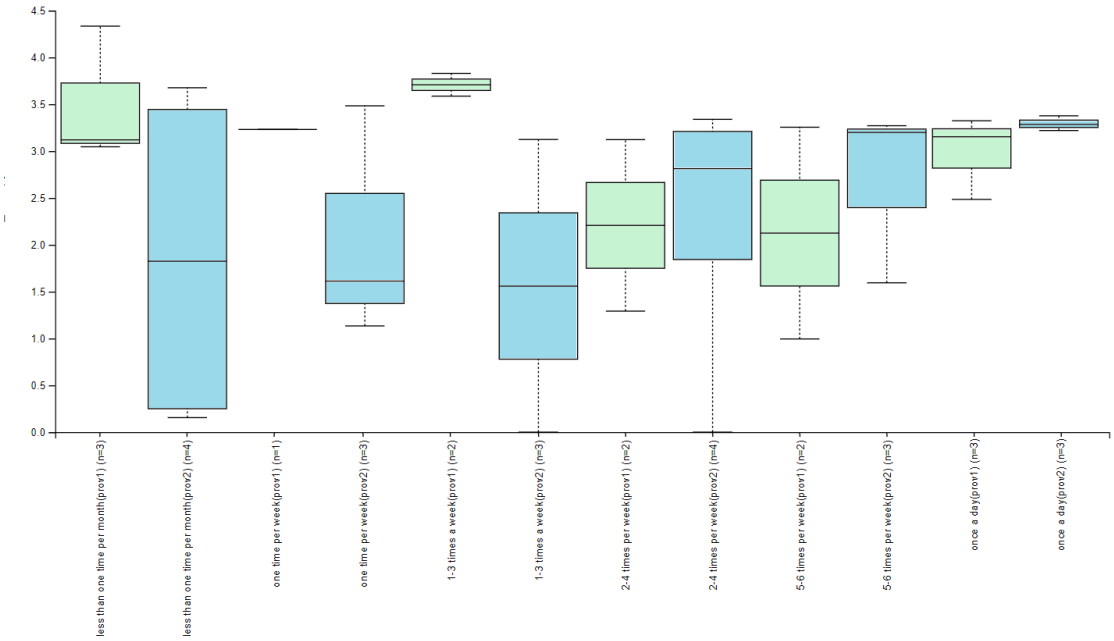

Figure 31. Boxplot charts depicting the distribution of the Shannon index in the different categories for frequency in wine tasting for each group (mean: Less than one time per month (prov1 – 3.5, prov2 – 1.9); One time per week (prov1 – 3.2, prov2 – 2.1); 1-3 times per week (prov1 – 3.7, prov2 – 2.3); 2-4 times per week (prov1 – 2.2, prov2 – 2.9); 5-6 times per week (prov1 – 2.1, prov2 – 3.2); once a day (prov1 – 2.9, prov2 – 3.3); p=0.48), prov1 samples in green and prov2 In blue

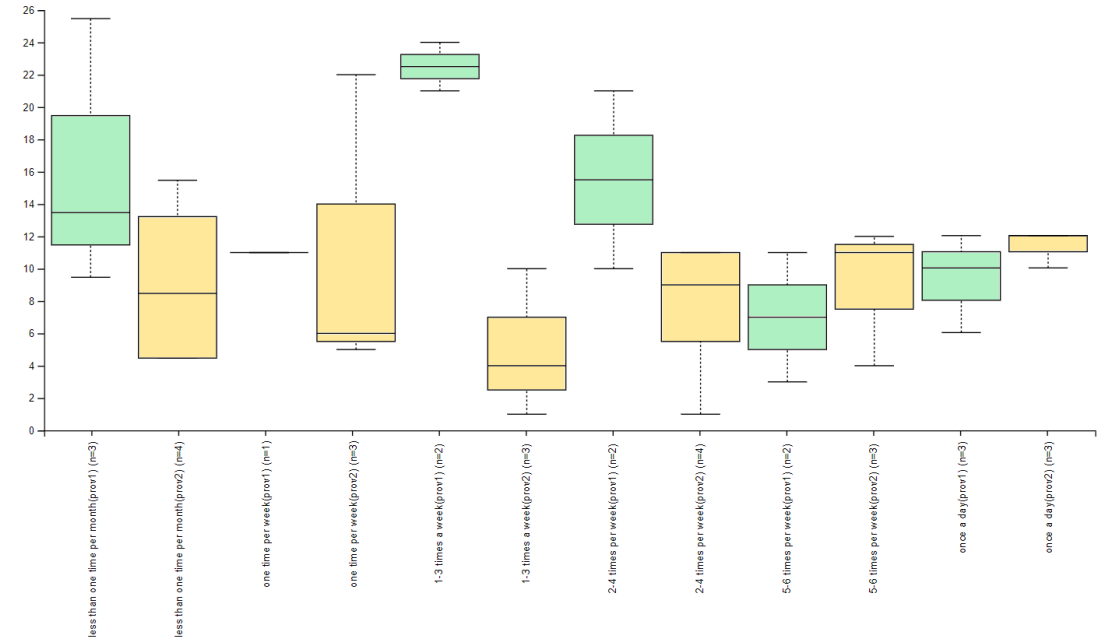

Figure 32. Boxplot charts depicting the distribution of the ASVs counts in the different categories for frequency in wine tasting for each group (mean: Less than one time per month (prov1 – 12.7, prov2 – 11.5); One time per week (prov1 – 2, prov2 – 5.3); 1-3 times per week (prov1 – 7, prov2 – 6); 2-4 times per week (prov1 – 14.5, prov2 – 14.5); 5-6 times per week (prov1 – 17.5, prov2 – 13.3); once a day (prov1 – 19.3, prov2 – 8.6); p=0.36), prov1 samples in green and prov2 in yellow

The comparison between variables such as age, smoking habits, alcohol consumption, oral hygiene habits such as teeth brushing, use of mouthwash, frequency of attendance to the dentist, and career length of wine taster were also compared using alpha-diversity measures (Supplementary Figures 5-20). The variable age was divided into three categories according to percentiles (0-33%; 33-67% and 67-100%). Category 1 (percentiles 0-33%) obtained the higher value of Shannon index between samples. When comparing the ASV counts the category 2 (percentiles 33-66%) obtained the higher value. The category of non-smoker had the higher value of Shannon index and ASV counts. Regarding alcohol consumption was divided into 4 categories according to the mean of frequency of consumption (less than one time per month, two times per week, more than two timer per week, at least one time per day) the category of consumption “two times per week” had the higher value of Shannon index and ASV counts. When comparing between groups, the category 3 of the control group obtained the highest value of Shannon index and ASV counts. The variables of oral hygiene habits, comparing the frequency of teeth brushing, category “2 times a day” had the higher value for Shannon index and ASV counts. Regarding the use of mouthwash elixir and frequency of attendance to the dentist, the individuals using mouthwash and going to the dentist “At least one time per year” had the highest values of Shannon index and ASV abundance.

4.2.5.2. *Beta* Diversity

Bray Curtis dissimilarity distances were calculated to measure the differences between individuals, to consider phylogeny it was calculated the unweighted frac with PERMANOVA. The beta diversity values between samples from the three different groups, and the distances to control, prov1 and prov2, respectively, are represented in Figure 33. According to the beta diversity values, there is a differentiation in the microbiome composition among the three groups (Pseudo-F = 1.67, p=0.025).

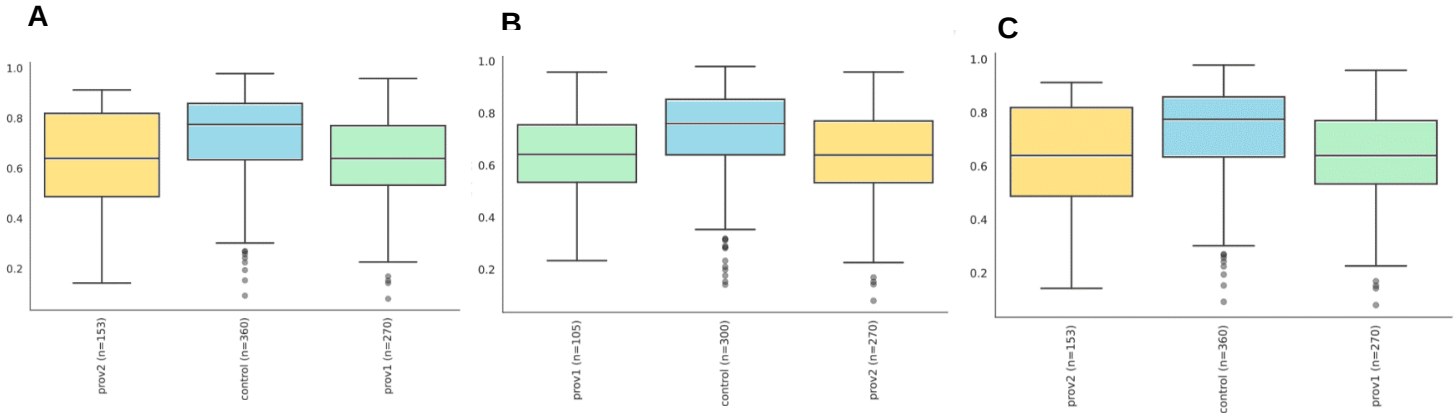

Figure 33. Unweighted frac values within each of the three groups and between them. (A) distance to control group. (B) distance to prov1. (C) distance to prov2. Control samples are given in blue, prov1 in green and prov2 in yellow

To see if there was any difference between the control group and both wine tasters' group collectively, it was calculated the Bray Curtis dissimilarity between these two groups. In Figure 34 is represented the distances to the control group and to prov1+prov2 group (Pseudo-F = 2.40, p= 0.01). The result demonstrated a difference in the composition of the oral microbiome of both groups.

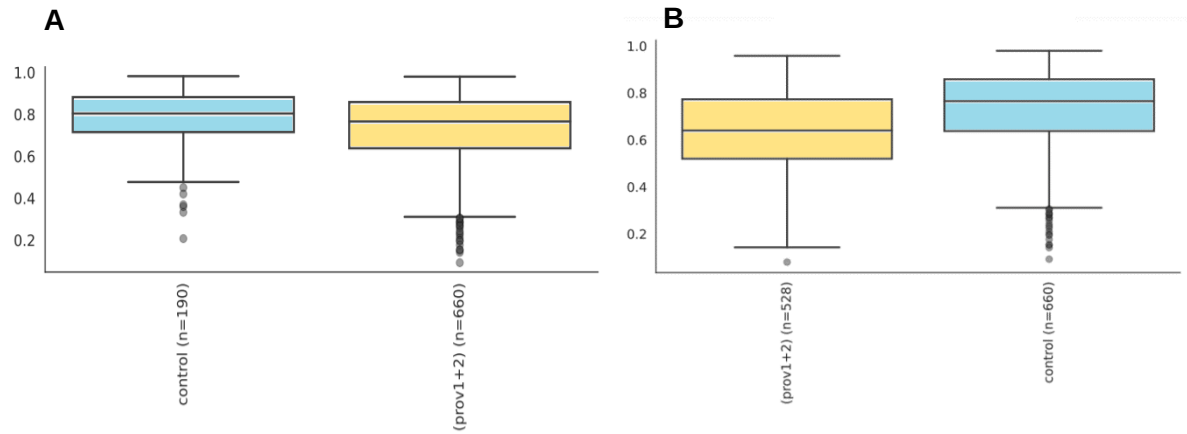

Figure 34. Unweighted frac values within each of the two groups and between them. (**A**) distance to control group. (**B**) distance to prov1+prov2 group. Control samples are colored in blue and prov1+ prov2 in yellow

Considering that the prov2 group, is the sample collected after a wine tasting, the effect of alcohol in the oral microbiome could be seen by comparing these samples with the other two groups. However, the Bray Curtis dissimilarity between prov2 group and control+prov1 group (Figure 35), did not show significant differences between the microbiome composition of both groups (Pseudo-F = 1.43, p = 0.128).

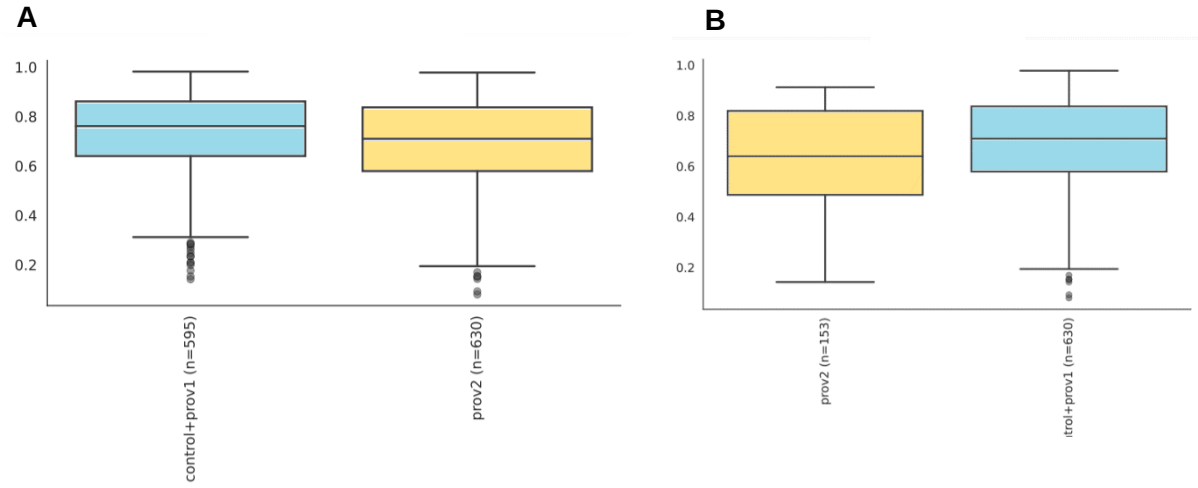

Figure 35. Unweighted frac values within each of the two groups and between them. (A) distance to control+prov1 group. (B) distance to prov2 group. Control+prov1 samples in blue, prov2 in yellow

The beta diversity distances between samples belonging to the same gender were different to the distances obtain when comparing samples of different gender (Pseudo-F = 1.75, p= 0.049). This permits to conclude that there is a significant difference in the microbiome composition between variable genders (Figure 36).

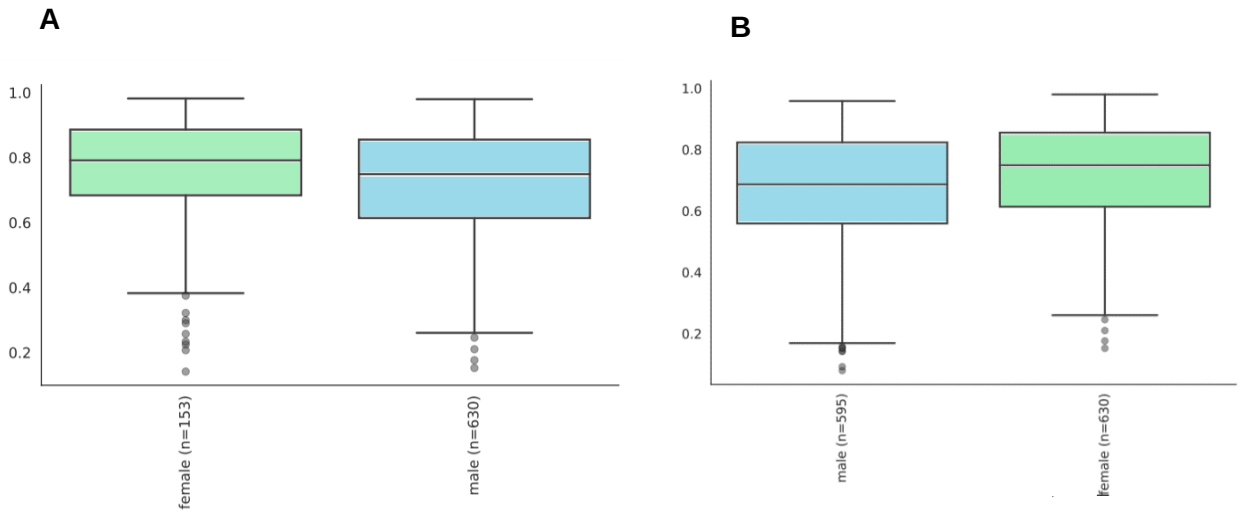

Figure 36. Unweighted frac values according to gender. (A) distance to female. (B) distance to male. Female samples in green, male in blue

A Principal coordinates analyses (PCoA) plot from the Bray-Curtis dissimilarity matrix was performed explore the taxonomic structure in the samples analyzed. The plot revealed a major aggregation between axis 2 and 3, formed mostly by samples from males from prov1 group. It is also possible to observe outside groups formed by some female samples belonging to control group (Figure 37).

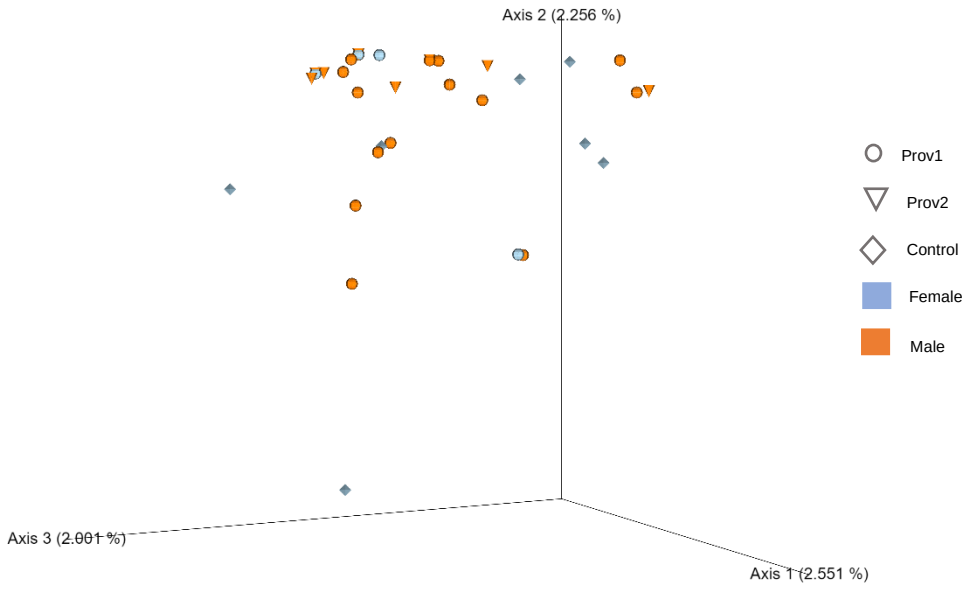

Figure 37. PCoA plot from Bray Curtis dissimilarity matrix. Percentage of the total variance represented by each axis is provided within parenthesis next to the label of the axis. Each shape represents one sample.

4.3. Characterization of the Diet, Lifestyle and Oral Hygiene Habits

For a better understanding of the habits of consumption of the different foods in each category of taste (sweet, sour, astringent, and bitter), the use of condiments, habits of oral hygiene including frequency of teeth brushing, attendance to the dentist and the use of mouthwash, all participants were invited to answer a questionnaire. Some variables such as the frequency of attendance in wine tastings, career time and care before tastings were only considered for the wine tasters' group. The consumption of alcohol and smoking habits were also included. For each of those variables mentioned before, their distributions between the two groups (control and wine tasters) were compared through a crosstab with a Chi-square of Pearson, with a 5% significance level. Considering the frequency of consumption of the sweet, bitter, astringent, and sour-taste foods, there was not significantly difference between groups ($p=0.392$; $p=0.906$; $p=0.667$; $p=0.792$). The tables with the counts for each group are represented in the Supplementary Tables 1 to 4.

When comparing the use of condiments between the two groups, there was not significantly difference between groups (Supplementary Table 5). The oral hygiene-related variables were compared between groups, for the "Frequency of teeth brushing" ($p=0.495$)," Attendance to the dentist" ($p=0.338$) and "Use of mouthwash" ($p=0.820$) there was no significant difference between groups. "Smoking habits" were also compared having no significant difference between groups ($p=0.979$) (Supplementary Table 6,7,8 and 9). The same variables were compared between gender, where the variables remain were not significantly different between genders (Supplementary Tables 10 to 13).

The variable "Frequency of attendance to wine tastings" between career lengths was also compared and demonstrated a significative difference ($p\leq0.001$), with wine tasters with more than 15 years of career have a higher frequency of attendance to wine tastings (Supplementary Table 14). It was also analyzed the frequency of tasting different types of wine between career length, the three types of wine considered were liqueur, sparkling and calm. The "Frequency of tasting liqueur wines" between different length of the career, was significantly different ($p\leq0.001$) (Supplementary Table 15) where the wine tasters with a career of 10 to 15 years demonstrated a higher frequency of tasting this type of wine. As also the "Frequency of tasting calm wines" ($p\leq0.001$) (Supplementary Table 16) and sparkling wines ($p=0.002$) (Supplementary Table 17), where the wine tasters with more than 15 years of career showed a higher frequency of consumption. The variable "Higher sensibility for taste" was compared between the two groups of wine tasters, not demonstrating a significative difference ($p=0.732$) (Supplementary Table 18).

4.3.1. Correlation between diet, lifestyle, oral hygiene, and the tongue dorsum microbiome

We have tested whether there was significant association between these variables and the tongue dorsum microbiome using data from the variables related to diet, oral hygiene, and career of wine tasters. For this purpose, the Spearman correlation was used with the different variables and the identified taxa from each database.

Greengenes database: Pasteurellaceae presented a positive correlation with the variable of wine taster career length (p=0.023). Regarding variables from the diet, Gemellaceae showed a negative correlation with the "Higher frequency of consumption of foods from bitter-taste related" (p=0.024). *Campylobacter* (p≤0.001)*, Fusobacterium* (p=0.004)*,* Weekcellaceae (p=0.003)*, Aggregatibacter* (p=0.027)*,* Pasteurellaceae (p≤0.001)*,* Gemellaceae (p=0.003) and Neisseriaceae (p≤0.001) presented a negative correlation with the variable of "Higher consumption of paprika". *The* Clostridiales (p=0.008) and Veillonellaceae (p=0.012) presented a negative correlation with the variables of "Higher consumption of mustard". Lactobacillalles (p=<0.001), Actinomycetales (p=0.011), *Neisseria* (p=0.022), *Fusobacterium* (p<0.001), *Porphyrmonas* (p≤0.001), *SR1* (p=<0.001), *Catonella* (p≤0.001), *Peptococcus* (p<0.001) and *Cardiobacterium* (p=<0.001) showed a negative correlation with the variable "Higher sensibility to sweet-taste". *Rothia* (p=0.003) presented a negative correlation with "Higher sensibility to bitter and sour taste". *Campylobacter* (p≤0.001) and *Veillonella* (p=0.026) showed a negative correlation with variable "Higher sensibility to sweet and sour taste". *Neisseria* (p≤0.001) presented a negative correlation with the variable "Higher sensibility to sweet and bitter taste". *Streptococcus* (p=0.004) showed a negative correlation with the variable "Equal sensibility to all tastes".

SILVA database: *Porphyromonas* showed a negative correlation with the variable "Higher frequency of consumption of foods sweet-taste related (p=0.034), and *Actinobacillus* (p=0.029), Weeksellaceae (p=0.029) and *Oribacterium* (p=0.029) presented a positive correlation with the same variable. A positive correlation was also presented between *Leptotrichia* (p=0.038) and the variable of "Frequency of attendance to wine tastings". *Lactobacillales* (p=0.022) presented the variable "Higher sensibility to sweet-taste". *Leptotrichia* (p=0.033) and *Lachnoanaerobaculum* (p=0.033) also presented a negative correlation with the variable "Higher sensibility to bitter-taste". Proteobacteria (p≤0.001) and *Haemophilus* (p=0.019) showed negative correlations with the variable "Higher sensibility to bitter and sour taste". *Neisseria* (p=0.022), *Campylobacter* (p=<0.001), *Fusobacterium* (p≤0.001), *Porphyromonas* (p≤0.001), *Cardiobacterium* (p≤0.001), *Peptococcus* (p≤0.001), *Absconditabacteriales* (p≤0.001),

Catonella (p≤0.001) and Carnobacyeriaceae (p≤0.001) presented a negative correlation with “Higher sensibility to sweet and sour taste”. *Micrococcales* (p≤0.001), *Haemophilus* (p=0.010) and *Burkholderiales* (p≤0.001) showed a negative correlation with the variable “Higher sensibility to sweet and bitter taste”.

HOMD database: *Stomatobaculum* presented a positive correlation with the variable “Frequency of consumption of sweet taste” (p=0.029), and *Leptotrichia* with the “Sour taste” (p=0.044). With the variable of consumption of paprika, a negative correlation was presented with the taxa Prevotellaceae (p=0.023), *Actinobacteria* (0.023), *Bacilli* (p=0.003) and *Fusobacterium* (p=0.023). *Catonella* (p≤0.001) showed a negative correlation with the variable “Higher sensibility to sweet-taste”. Lachnospiraceae (p=0.033) and *Leptotrichia* (p=0.033) presented a negative correlation with the variable “Higher sensibility to bitter-taste”. *Streptococcus* (p=0.004) and *Veillonella* (p=0.016) showed a negative correlation with the variable “Equal sensibility to all tastes”.

4.4. Characterization of the Nasal Microbiome

Regarding the nostril samples, the total number of raw reads was 3,807,196. The minimum number of reads per sample was 190,249 and the maximum was 512,821 reads. With a mean of 346,108.7 reads per sample. After quality filtering, 3,678,933 reads remained. The two groups showed a similar average number of reads per individual, the tasters’ group 321,208 reads, and the control group 345,482 reads (Figure 38).

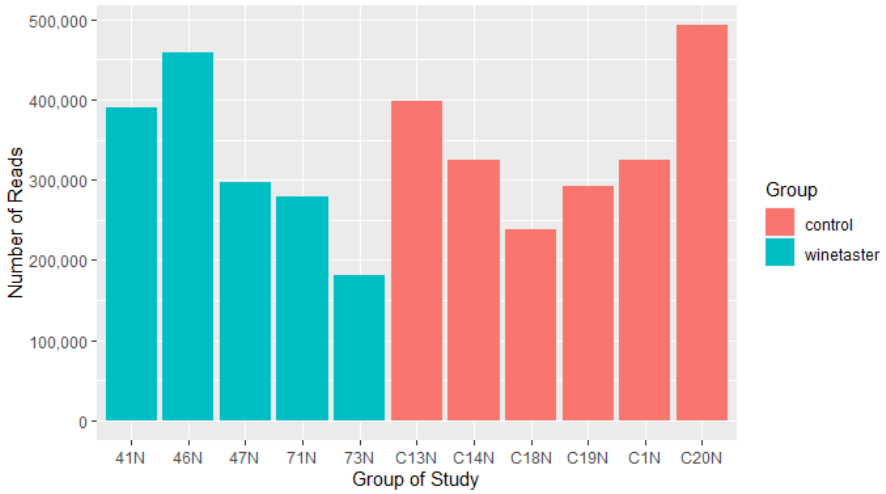

Figure 38. Bar chart representing the number of reads per individual and per group (Nasal Samples) – control samples in red and wine taster samples in blue.

In the 11 nostril samples a total of 218 ASVs were observed, from a total frequency of 288,522 reads. The mean frequency of ASVs per sample was 30,572 (range: 189 – 60,676). After sampling rarefaction 19,450 reads (6.74%) and 10 samples (90.91%) were retained. Five samples were preserved from the control and wine taster group, only one sample was excluded from the control group, evening the number of samples between the two groups. For nostril samples, the ASVs alignment was performed with the same databases as with the oral samples. The Greengenes databased assigned 22 taxa to the ASVs of the nostril samples, the SILVA 19 taxa, the RDP eight, and the HOMD database five taxa.

4.4.1. Comparison Between Groups

***Greengenes database*:** the wine tasters and control group shared 9 tout of the 22 OTUs. In the control group were observed 10 OTUs, which were absent in the wine tasters' group. Whereas the wine tasters group presented three OTUs that were absent from the control group.

***SILVA database*:** The two groups shared seven out of the 19 OTUs. The control group showed nine OTUs that were absent in the wine tasters' group, whereas the latter one displayed three OTUs that were absent in the first group.

HOMD database: only 5 OTUs were identified, from which two were shared among groups, while the other three were only present in the control group.

RDP database: From a total of eight OTUs identified, three were only present in the control group and the other five were shared by the two groups.

4.4.2. Taxonomic classification of the taxa

Greengenes database: More than half of the samples were only possible to identified as Bacteria (57%) and 39% of the samples were Unassigned. The remain four percent were identified as Actinobacteria (3%) and Proteobacteria (0.3%). Frequencies of the ten more representative OTUs are represented in Figure 39A.

SILVA database: 82% of the taxa present in the samples Unassigned and 14% were identified as Bacteria. Three phyla were identified Actinobacteria (3%), Proteobacteria (0.2%), and Firmicutes (0.01%). Frequencies of nine more representative OTUs are represented in Figure 39B.

RDP database: Only one percent was Unassigned, with Eukaryarchaeota (84%) dominating, following by Proteobacteria (1%). 15% were identified only at domain level

as Archaea. Frequencies of the four more abundant OTUs are represented in Figure 39C.

HOMD database: 99% of taxa present in the samples were Unassigned. The remain one percent were Actinobacteria (0.09%), Proteobacteria (0.03%) and Firmicutes (0.000006%). Frequencies of the four more representative OTUs between samples are represented in Figure 39D.

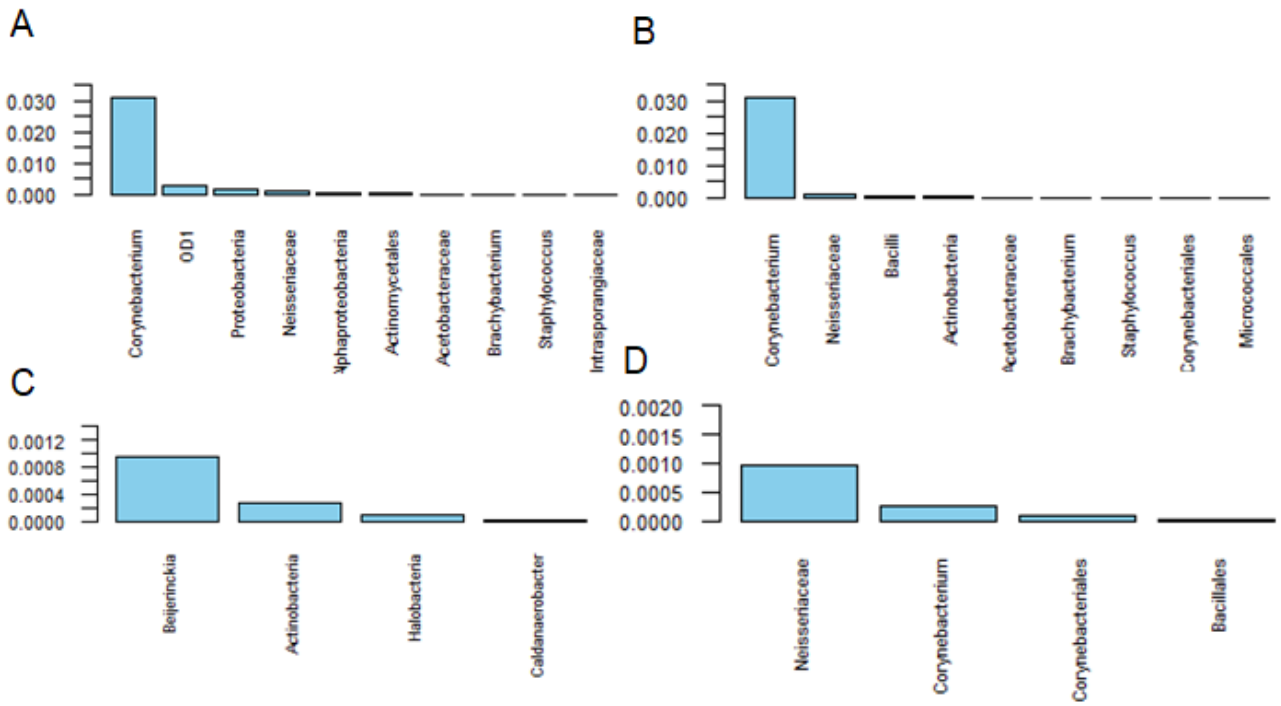

Figure 39. Bar plot representing the frequencies of the most representative OTUs. (**A**) Greengenes, (**B**) SILVA, (**C**) RDP, (**D**) HOMD databases

4.4.3. Distribution and abundance of the main taxa

As before, after sequence processing and alignment, the nasal samples were compared to four databases.

Greengenes database: It were identified 4 phyla, 5 classes, 9 orders, 13 families, 7 genera and 2 species. The mean number of OTUs per individual is 6. The most abundant OTUs belong to *Corynebacterium* and *OD1* genera (Figure 40).

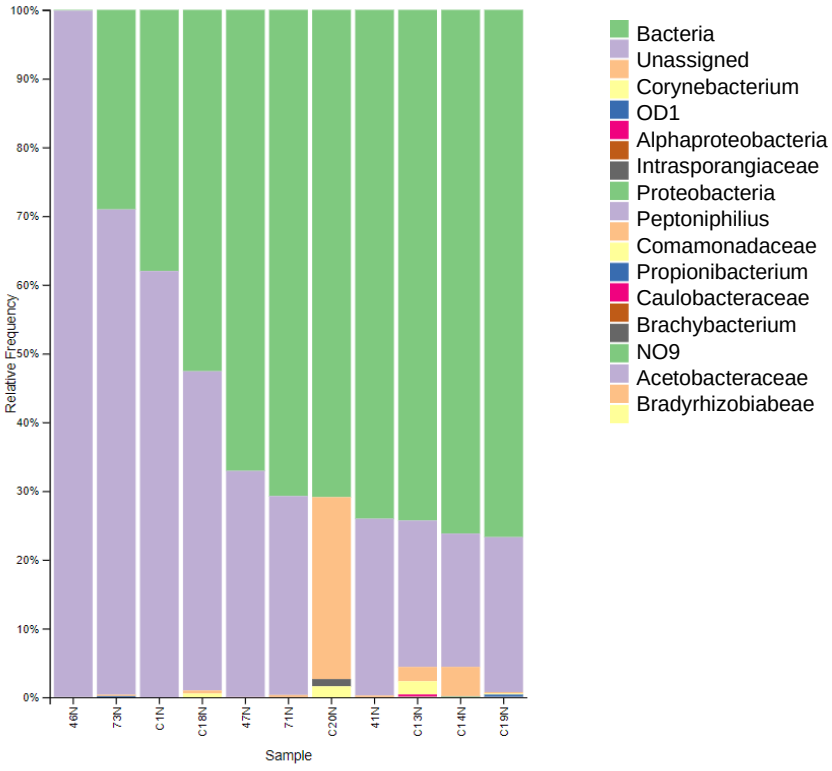

Figure 40. OTUs observed at each sample (up to the genus level) identified in Greengenes database. Sample names starting with C are individuals from the control group and those ending in N are individuals from the wine tasters' group.

SILVA database: Identified 4 phyla, 5 classes, 10 orders, 11 families, 7 genera and 4 species. The mean number of OTUs per individual was 5. The most abundant OTUs belong to the *Actinomyces* and Pasteurellaceae genera (Figure 41).

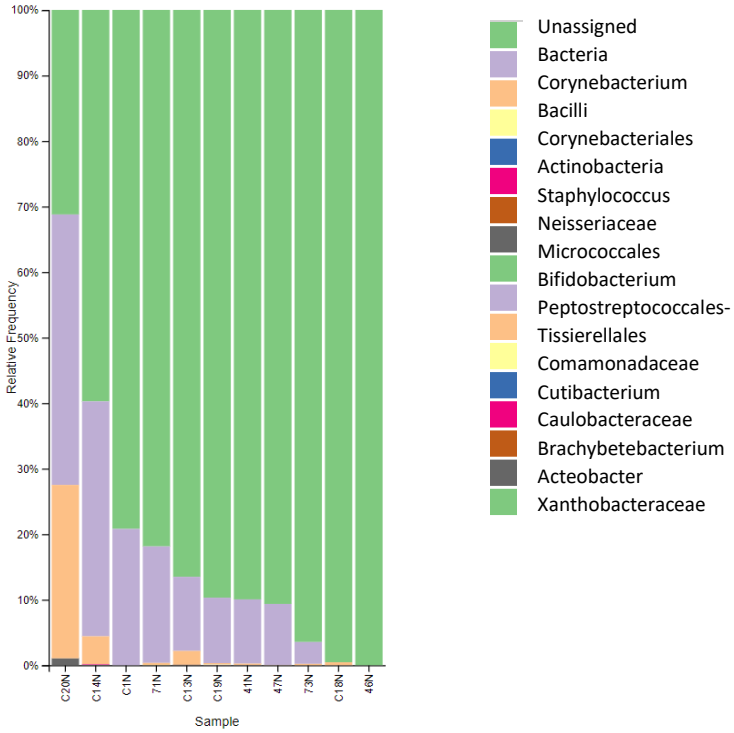

Figure 42. OTUs observed at each sample (up to the genus level) identified in SILVA database. Sample names starting with C are individuals from the control group and those ending in N are individuals from the wine tasters' group

RDP database: Identified 4 phyla, 4 classes, 2 orders, 2 families, 7 genera and 2 species. The mean number of OTUs per individual was 3. The most abundant OTUs up to the genus level were *Beijerinckia* and Actinobacteria (Figure 42).

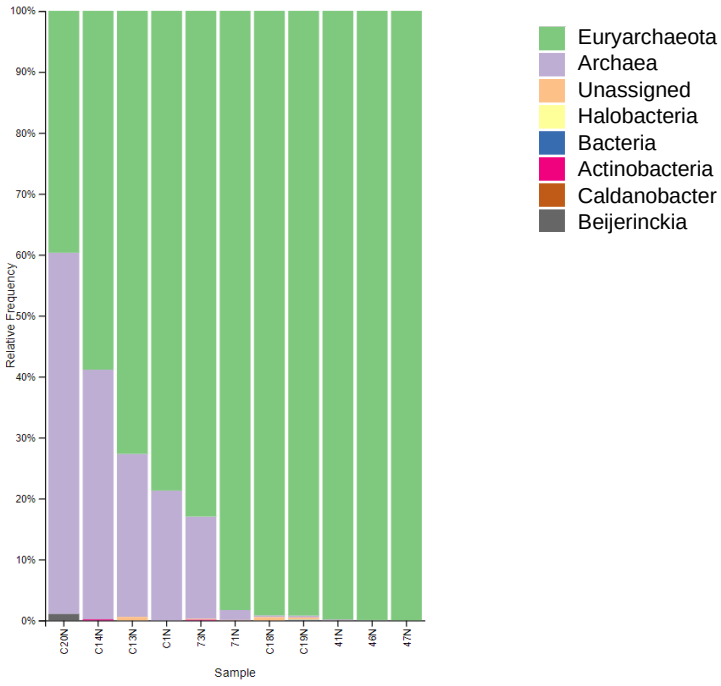

Figure 41. OTUs observed at each sample (up to the genus level) identified in RDPdatabase. Sample names starting with C are individuals from the control group and those ending in N are individuals from the wine tasters' group

HOMD database: The database HOMD was excluded for further analysis since provide less information regarding the identification of taxa when comparing with the other databases. Nonetheless, it was possible to identify 3 phyla, 3 classes, 3 orders, 2 families, 2 genera and 1 specie. The mean number of taxa per individual is 2. Neisseriaceae and *Corynebacterium* were the most abundant OTUs at the genus level.

A heatmap for each selected database was built with taxa relative abundance and the top 50 taxa most abundant in the samples The ASVs identified as "Unassigned" and *Bacteria* were excluded to achieve a clearer view of the identified taxa in the samples.

Greengenes database: the most abundant OTUs were from *Corynebacterium genus*, no shared OTU among individuals was observed. There were two major clusters formed based on the relative frequency of *Corynebacterium* (Figure 43). Individuals from the control and wine tasters' groups are present in both clusters.

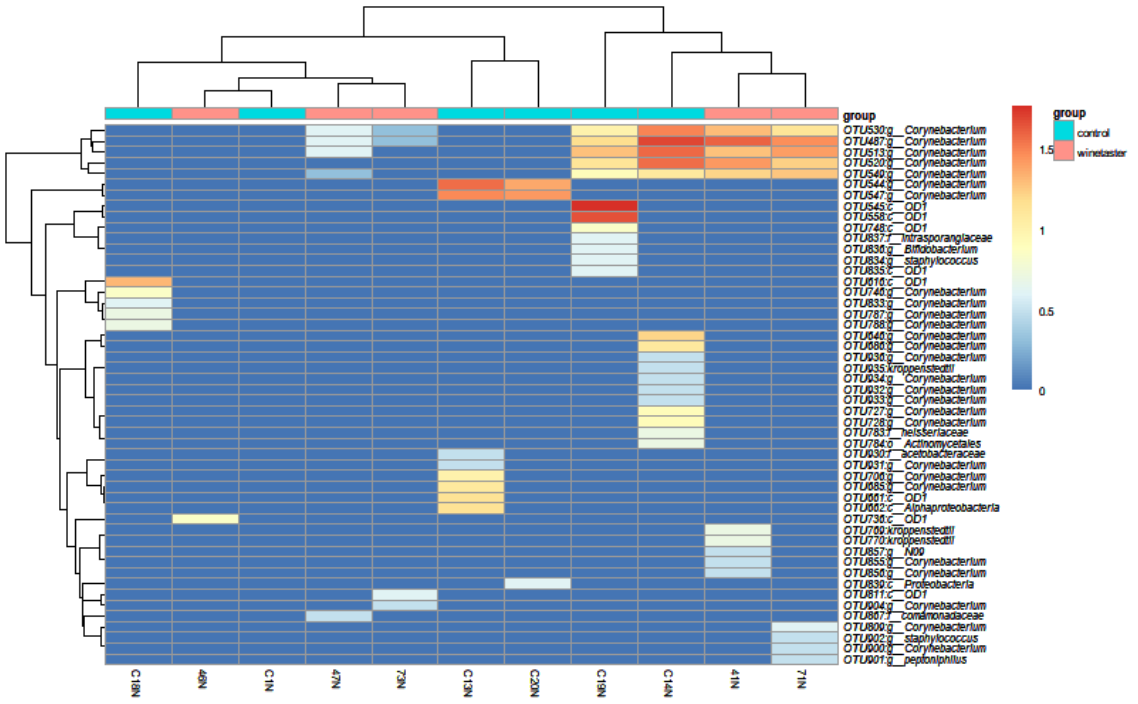

Figure 43. Top 50 most abundant OTUs up to the genus level per subject. Sample names starting with C are from the control group and those ending in D or A are from the wine taster group – Greengenes. Control group in blue color, and wine tasters in red color.

SILVA database: Considering the 50 most abundant OTUs, the most abundant was the *Corynebacterium,* in agreement with the previous database. There were two major clusters coincident with the previous heatmap, based on the relative abundance of *Corynebacterium (*Figure 44).

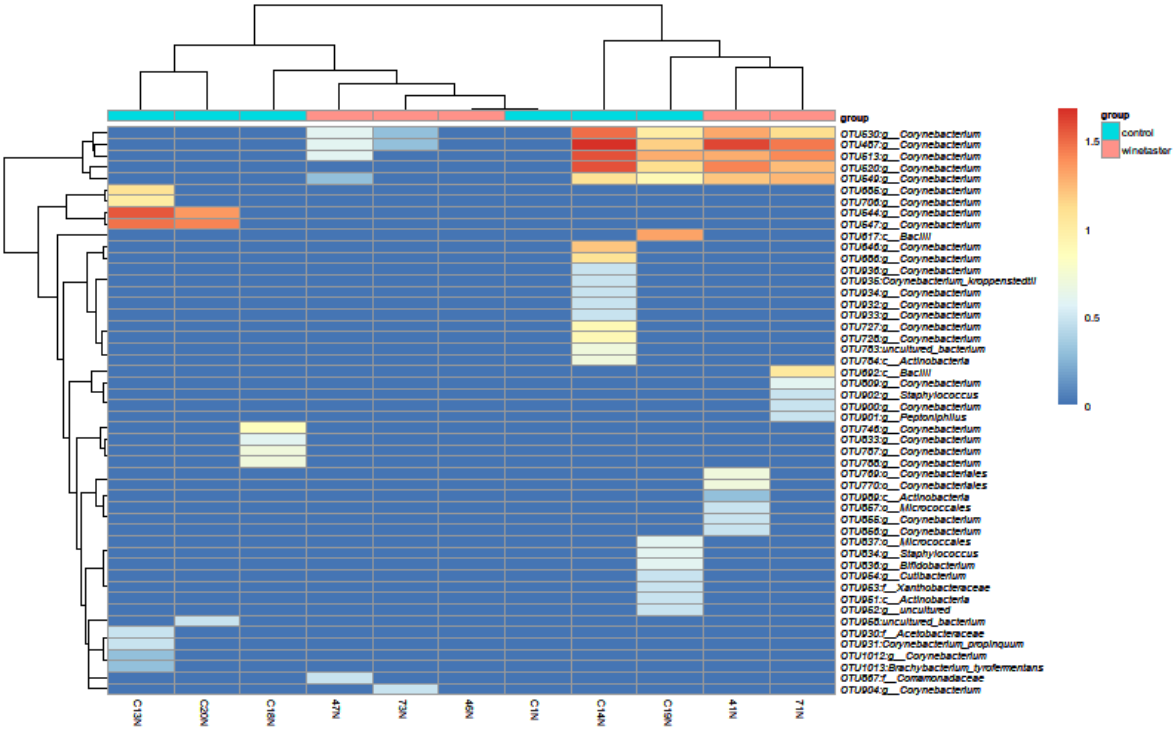

Figure 44. Top 50 most abundant OTUs up to the genus level per subject. Sample names starting with C are from the control group and those ending in N are from the wine taster group – SILVA. Control group in blue color, and wine tasters in red color

***RDP database*:** The most abundant taxa was the *Corybacterium* as the previous two databases. The two clusters are based on the relative abundance of *Corybacterium* with individuals from the two groups (Figure 45).

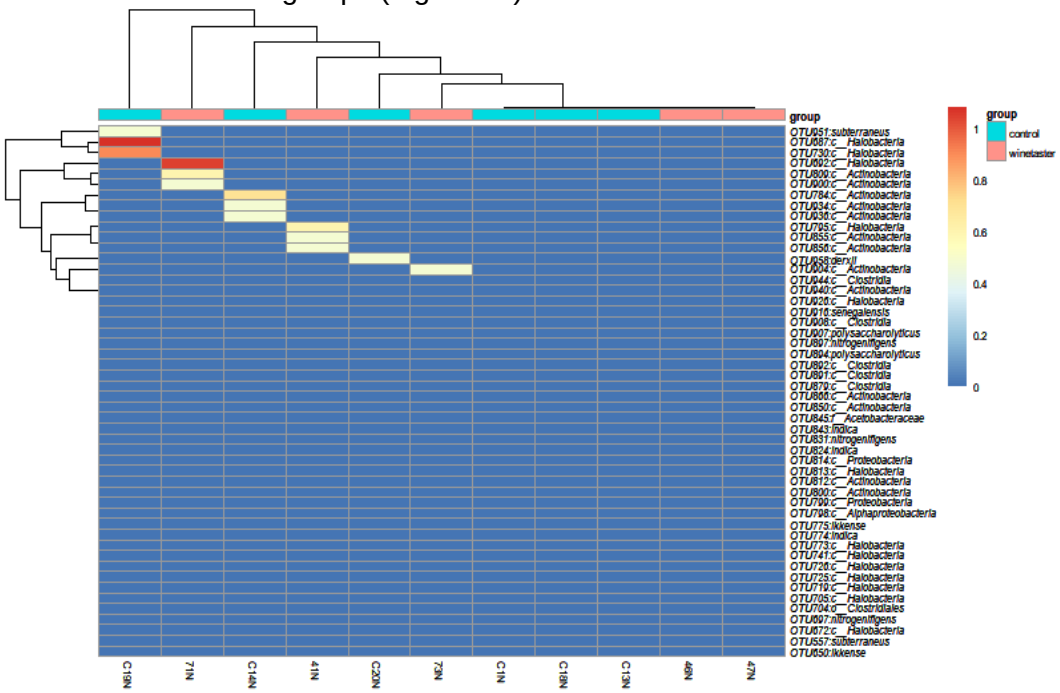

Figure 45. Top 50 most abundant OTUs up to the genus level per subject. Sample names starting with C are from the control group and those ending in N are from the wine taster group – RDP. Control group in blue color, and wine tasters in red color.

4.4.4. Microbiome differences between groups

To assess the differences in microbial taxonomy between control and wine taster group, the bacterial composition was compared at class and genus levels with Kruskal-Wallis Test. The taxa defined as Unassigned and *Bacteria* were excluded. The test was performed in Greengenes, RDP and SILVA databases, and all demonstrated that was no significant difference between the two groups at the class or genus level.

4.4.4.1. *Classes*

Relative abundances

The three databases identified an average of 97% of the taxa present in the samples of both groups has Unassigned or only identified as Bacteria, not identifying phyla. Since this does not add relevant information to the study, this percentage of taxa was excluded.

Greengenes database: The percentage of excluded varied between groups, being 94% for the control group and 99% of the wine tasters' group. From the 7 classes identified showed in Figure 46, Actinobacteria is the dominant class in the two groups, with the relative abundance of 6% in the control group and 0.16% in the wine tasters' group. The main differences are the higher abundance of OD1 (0.4%), Proteobacteria (0.3%), Betaproteobacteria (0.2%) in the control group. *Clostridia* (0.001%) is the most abundant in the wine tasters' group.

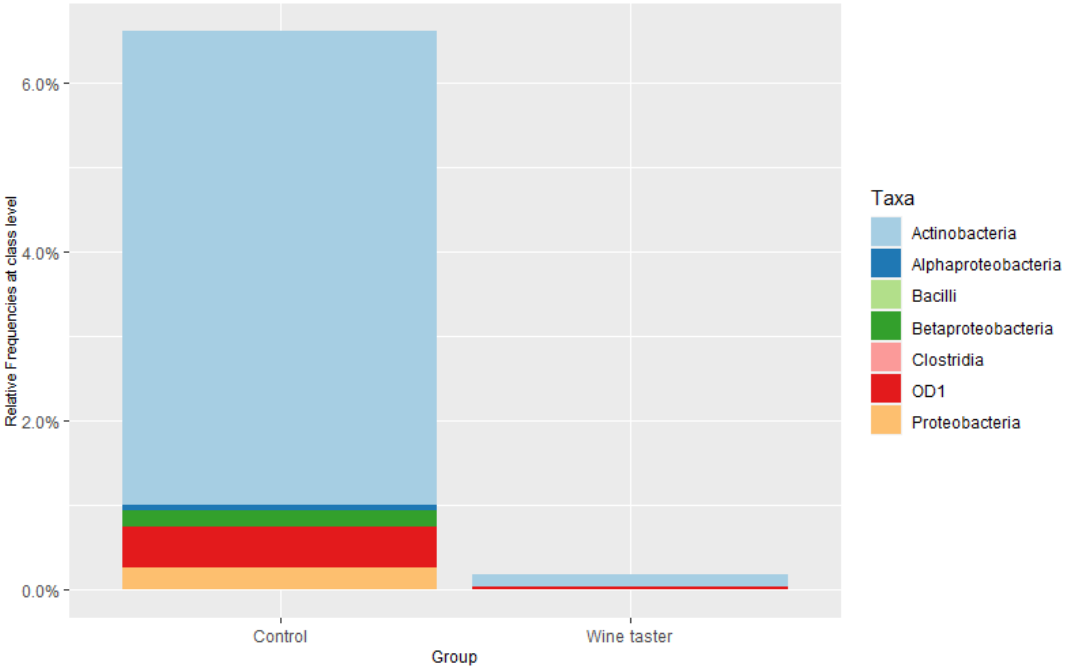

Figure 46. Classes identified with Greengenes database. OTUs identified as Unassigned, and Bacteria were excluded

Silva database: The percentage excluded from each group varied, being 93% for the control group and 99% of the wine taster group. From the 7 identified classes showed in Figure 47, *Actinobacteria* is the dominant class in both groups, with a relative abundance of 6% in the control group and 0.16% in the wine tasters' group. The classes Alphaproteobacteria (0.01%), Bacilli (0.01%) and Gammaproteobacteria (0.19%) are more abundant in the control group. On the other hand, Clostridia (0.001%) presents a higher abundance in the wine tasters' group.

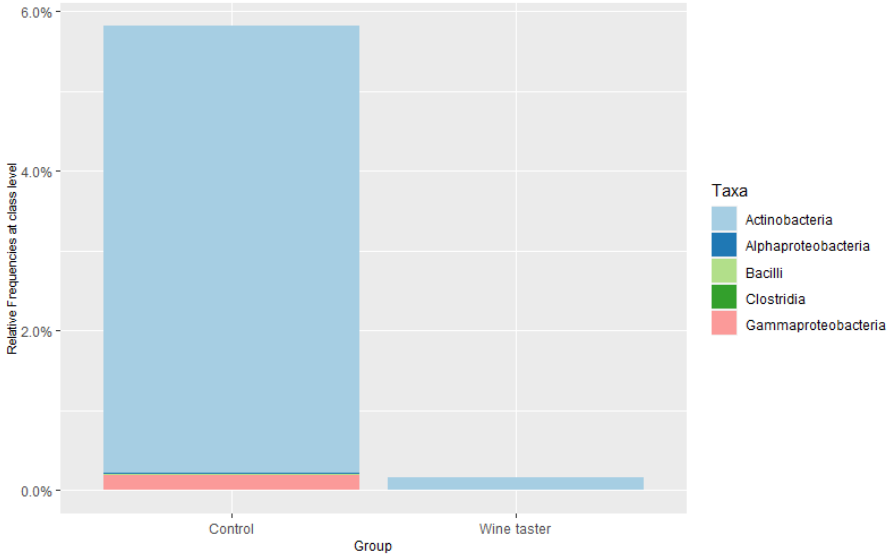

Figure 47. Classes identified with SILVA database. OTUs identified as Unassigned and Bacteria were excluded

RDP database: The percentage of taxa excluded varied between groups, being 98% for the control group and 99% for the wine taster group. From the three identified classes showed in Figure 48, Actinobacteria is the class present in both groups, with a relative abundance of 0.0003% in each group. Alphaproteobacteria is the most dominant class, but only present in the control group. Clostridia (0.001%) is more abundant in the control group.

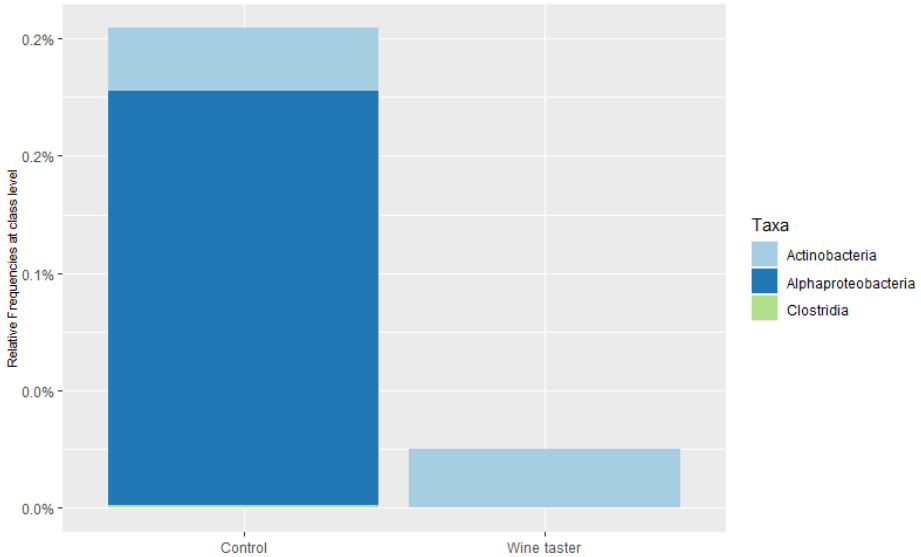

Figure 48*. Classes identified with RDP database. OTUs identified as Unassigned and Bacteria were excluded*

4.4.4.2. Genus

Relative abundances

Greengenes database: Some of the taxa was only possible to identify up to the class or family level. From the 10 most frequent genera of each group showed in Figure 49, *Corynebacterium* is the dominant genus in both groups, with a relative abundance of 6% in the control group and 0.15% in the wine tasters' group. The genera Alphaproteobacteria (0.05%), *Brachybacterium* (0.01%) and *Actinomycetales* (0.01%) are more abundant in the control group. The wine tasters' group had higher abundance of N09 (0.0007%) and *Peptoniphilus* (0.001%).

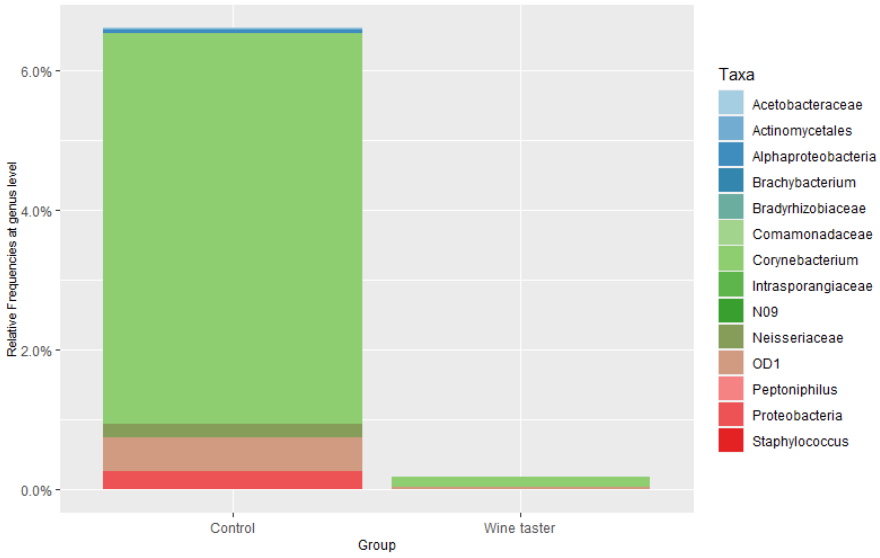

Figure 49. Genera identified with Greengenes database. OTUs identified as Unassigned and Bacteria were excluded

SILVA database: In consistency with the previous database some of the taxa was only possible to identify up to the class or family level. From the 10 most frequent taxa of each group showed in Figure 50, *Corynebacterium* is the most abundant genus in both groups, with a relative abundance of 6% in the control group, and 0,15% in the wine tasters' group. Neisseraceae (0.19%), Xanthobacteraceae (0.001%) and Bacilli (0.011%) were more abundant in the control group. The genera Acetobacteria (0.008%), *Brachybacterium* (0.008%), *Staphylococcus* (0.002%), *Micrococcales* (0.002%) and *Bifidobacterium* (0.002%) had a higher abundance in the control group.

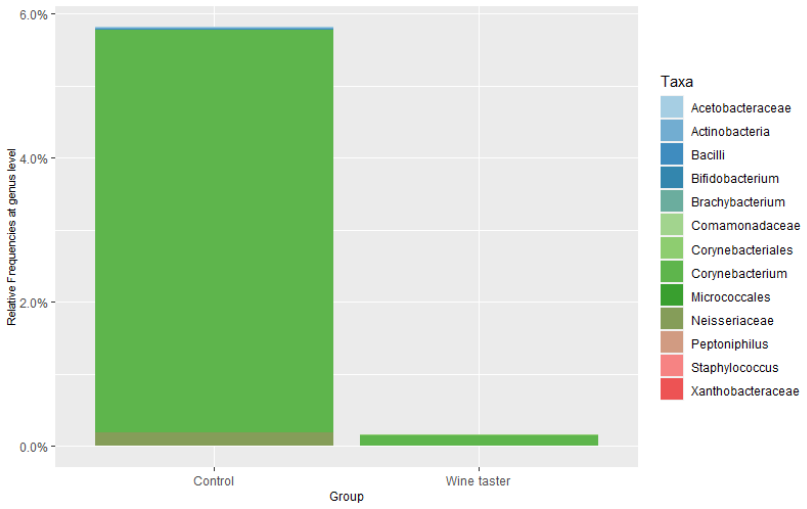

Figure 50. *Genera identified with SILVA database. OTUs identified as Unassigned and Bacteria were excluded*

RDP database: Again, some of the taxa was only possible to be identified at the phyla and class level. From the identified taxa showed in Figure 51, Actinobacteria is the taxa present in both groups, as showed in the previous figure, with the relative abundance of 0.027% in the control group and 0.025% in the wine tasters group. *Beijerinckia* (0,2%), *Caldanaerobacter* (0.001%) and Halobacteria (0.01%) had higher abundance in the control group.

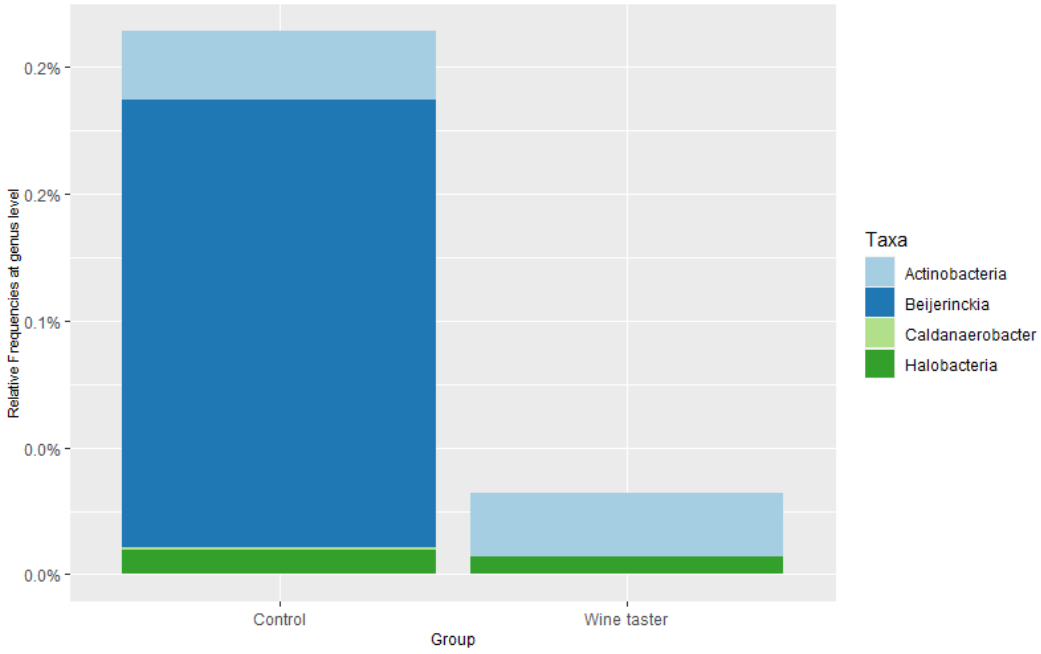

Figure 51. *Genera identified with RDP database. OTUs identified as Unassigned and Bacteria were excluded*

4.4.5. Microbiome Diversity

The sampling depth was selected before the assessment of the diversity measures. After, it was performed rarefaction curves for the Shannon index and ASV abundance, per group. The rarefaction curves for the Shannon index showed a plateau over 2500 sequence reads (Figure 52). Regarding the rarefaction curves for the ASV abundance, the one from the control group progressively approached to saturation, reflecting the species richness. The curve of the wine tasters' group has fewer richness of species, because the plateau is not yet reached. The samples were rarefied to 1945 reads, which is approximately the plateau value, and keep the maximum number of samples and ASV abundance.

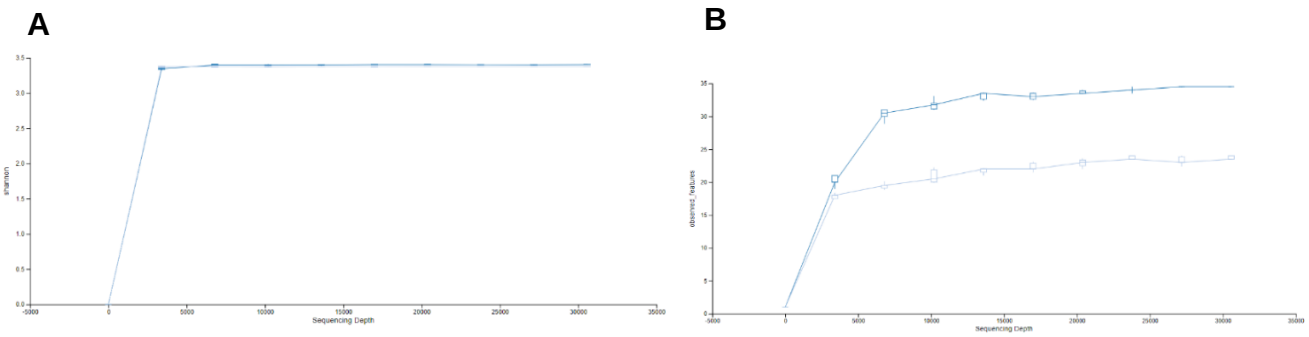

Figure 52. Rarefaction curves from (A) Shannon index and (B) the ASV abundance per group. The light blue represents the control group and blue the wine tasters' group

4.4.5.1. Alpha Diversity

Shannon index and ASVs abundance are the measures used for the alpha diversity, which were compared between groups, and different categories of the following variables: gender, age, alcohol consumption, frequency of consumption of food from the tastes sweet, bitter, sour, and astringent and wine taster career. Variables such as consumption of taste sweet, bitter, sour and astringent were divided into 5 categories, according to the frequency of consumption. Observing the Shannon index (Figure 53) it does not appear to have a significant difference between groups, which is supported by the p-value of Shannon index (p=0.92). The comparison between the ASV abundance (Figure 54) appears to be different but it is significantly different (p=0.28).

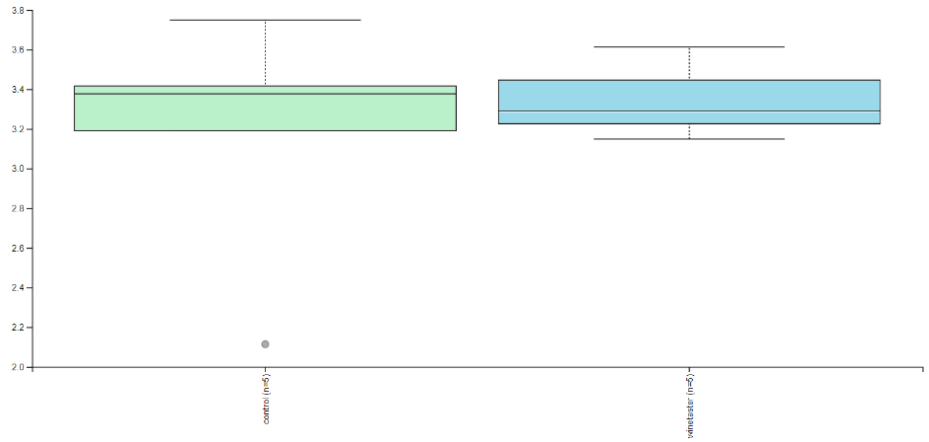

Figure 533. Boxplot charts depicting the distribution of the Shannon index between both groups (mean: control (3.2), wine taster (3.3)), control samples in green, wine tasters in blue

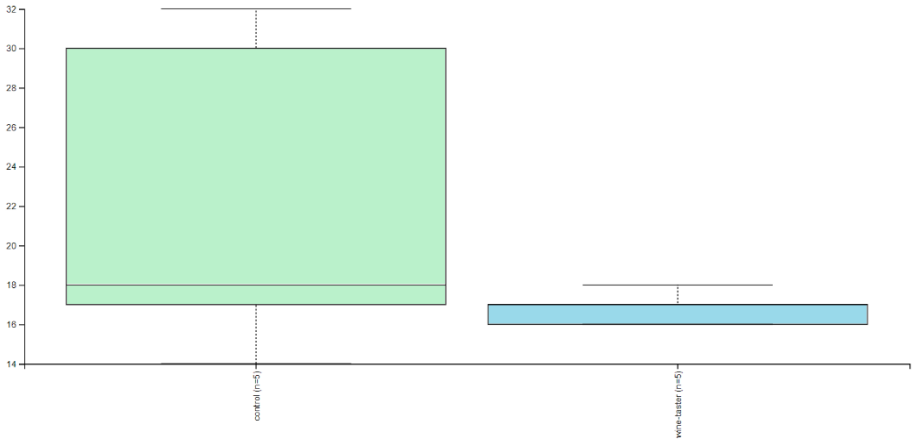

Figure 54. Boxplot charts depicting the distribution of the ASVs abundance between both groups (mean: control (22.2), wine taster (16.8)), control samples in green and wine tasters in blue

Regarding gender, the Shannon index and the ASVs abundance were calculated first for all the individuals and then distinctly according to the group. The Shannon index (Figure 55) between the two genders is not significantly different (p=0.60), for the gender according to the group (Figure 56) was also not significantly different (p=0.39).

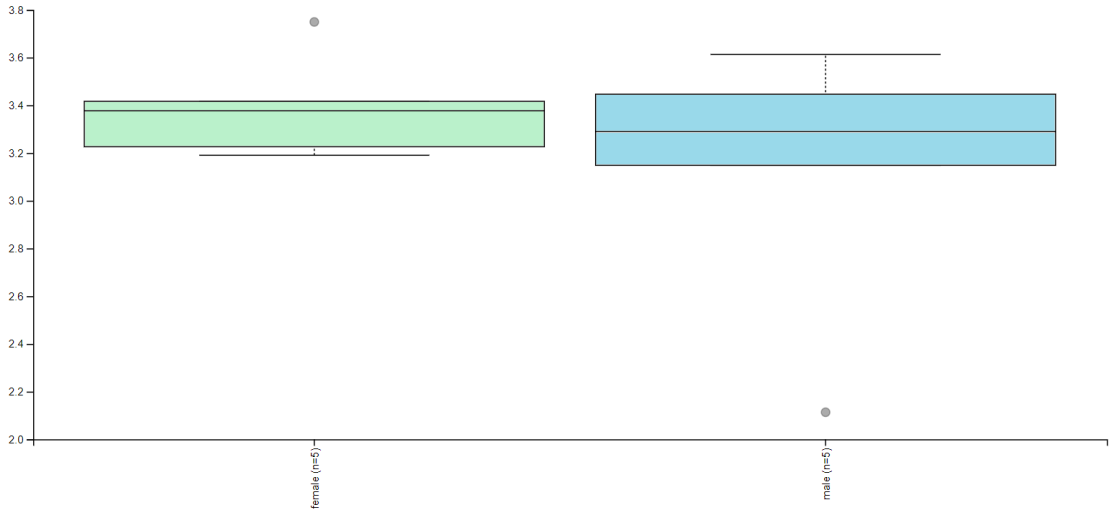

Figure 54. Boxplot charts depicting the distribution of the Shannon index between genders (mean: female (3.4), wine tasters (3.1)), female samples in green and males in blue

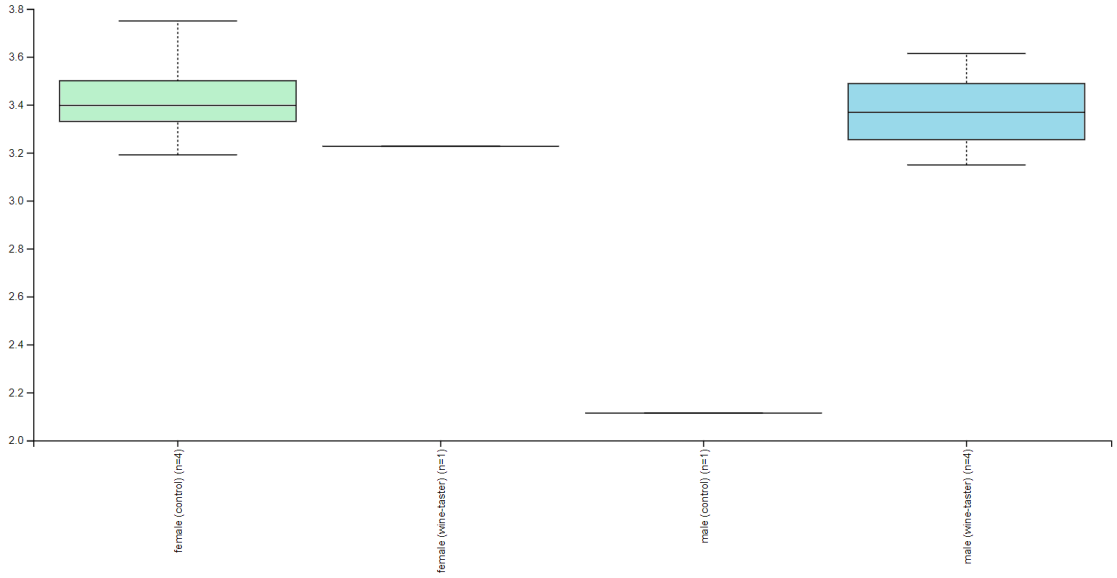

Figure 55. Boxplot charts depicting the distribution of the Shannon index between genders of both groups (mean: wine taster (female (3.2), male (3.4); control (female (3.4), male (2.1)), female samples in green and males in blue

The comparison of the ASV abundance between genders (Figure 57) demonstrated lower counts in males, but it was not significantly different (p=1.00). Likewise, when dividing the gender according to the groups (p=0.61 – Figure 58).

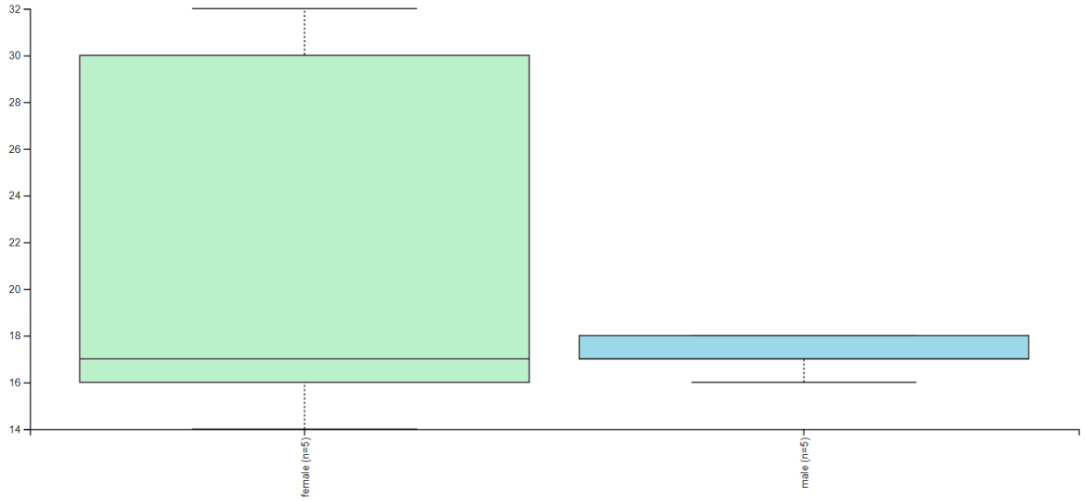

Figure 56. Boxplot charts depicting the distribution of the ASV counts between genders (mean: female (21.8) and male (17.2)), female samples in green and males in blue

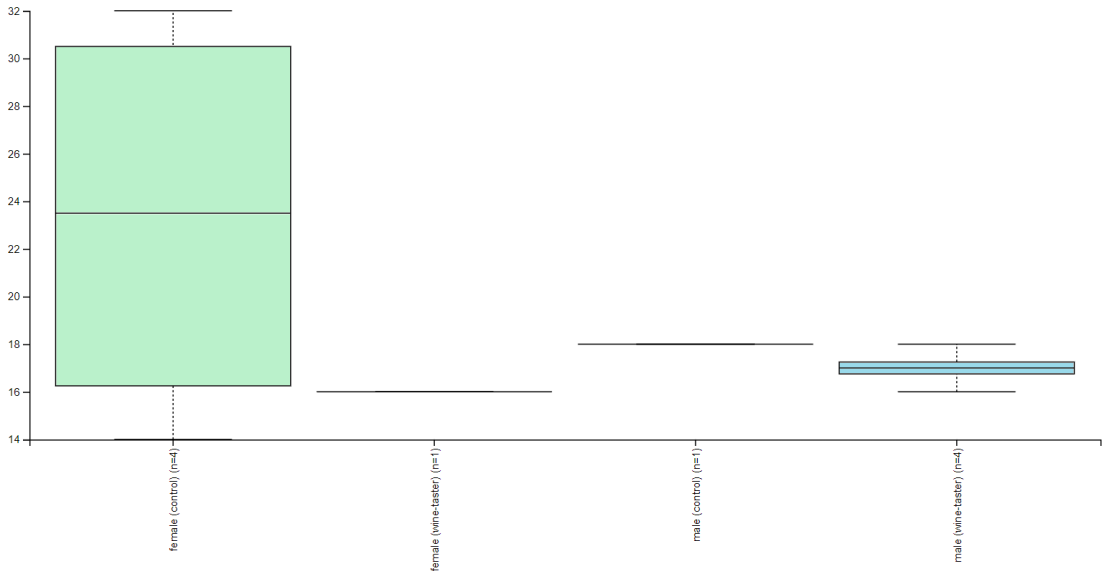

Figure 57. Boxplot charts depicting the distribution of the ASV abundance between genders separated by groups (mean: wine taster (female (16), male (17); control (female (23.3), male (18)), female samples in green and males in blue

The alpha diversity measures were also compared grouping the variables by categories of consumption of each taste, considering the control and wine tasters group separated. For every comparison there was no significant difference between groups and categories, so the variables will be represented in Supplementary Figures 21 to 28.

The variable “Frequency of alcohol consumption” between group was compared by Shannon index and ASV abundance, in none of the measures the result was significantly

different. The category of frequency “one time per day” showed the highest value of Shannon index and ASV counts, and the lowest Shannon index. The lowest ASV abundance were obtain by the categories of consumption of “1 to 3 times per week” from the control group and more than “one time per day” from the wine tasters’ group.

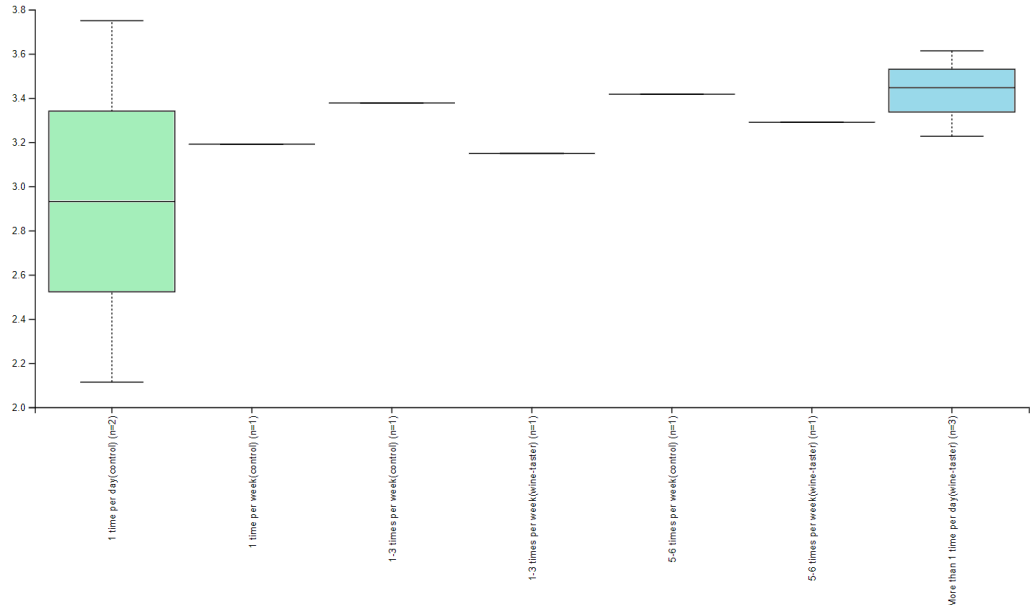

Figure 58. Boxplot charts depicting the distribution of the Shannon index in the different categories of alcohol consumption for each group (mean: control (1 time per day – 2.9, 1 time per week – 3.1, 1-3 times per week – 3.4, 5-6 times per week – 3.4); wine taster (1-3 times per week – 3.1, more than 1 time per day – 3.4, 5-6 timer per week – 3.3); p=0.80, control samples in green and wine tasters’ in blue.

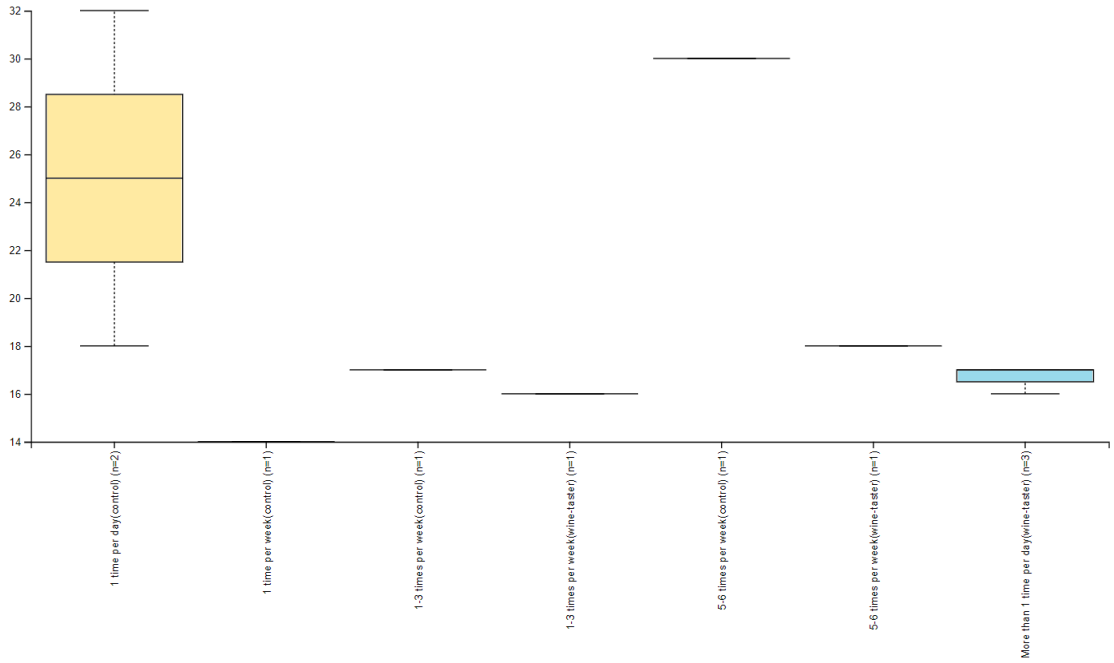

Figure 59. Boxplot charts depicting the distribution of ASV abundance in the different categories of alcohol consumption for each group (mean: control (1 time per day – 25, 1 time per week – 14, 1-3 times per week – 17, 5-6 times per week – 30); wine taster (1-3 times per week – 16, more than 1 time per day – 17, 5-6 timer per week – 18); p=0.22, control samples in orange and wine tasters’ in blue.

Regarding the group of wine tasters, the alpha diversity for the variable of career time it was not possible to calculate, because only one group was formed, all the subjects had the same duration of career time. When comparing the “Frequency of attendance in wine tastings” between the group of wine tasters, the category of frequency one time per week obtained the higher Shannon index, and “1 to 3 times per week” the lowest value of Shannon index and ASV abundance (Figure 61). The higher abundance of ASV were obtained by the category of frequency “one time per day” (Figure 62).

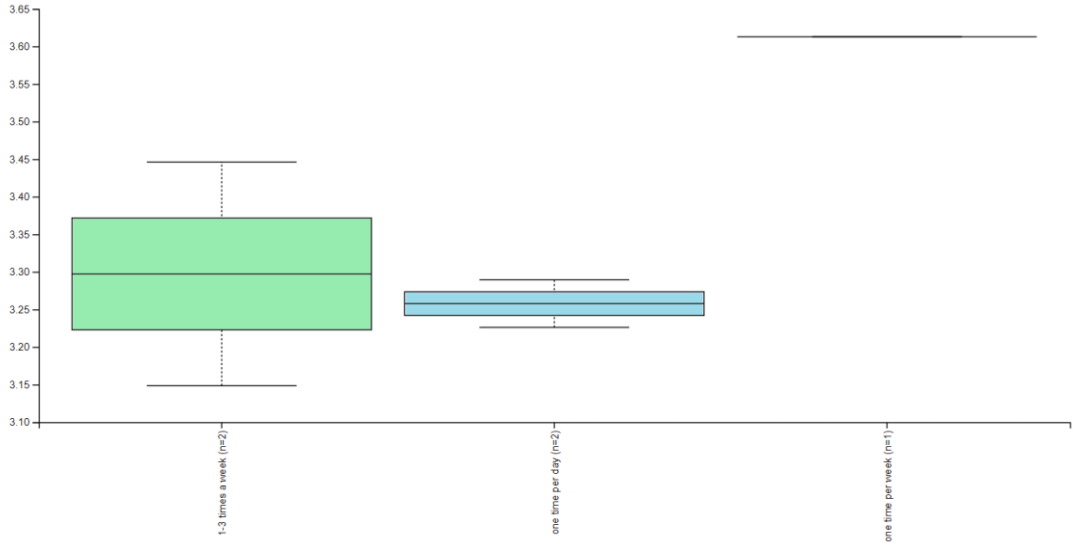

Figure 60. Boxplot charts depicting the distribution of the Shannon index in the different categories of wine tasting attendance for each group (mean: 1-3 times a week – 3.3, one time per day – 3.3, one time per week – 3.4); p=0.37.

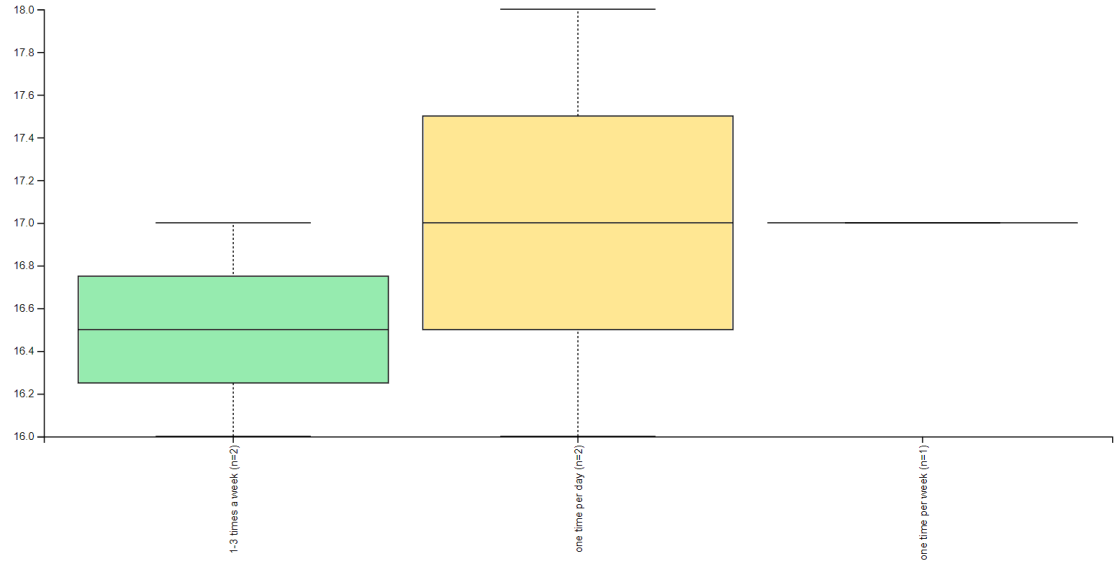

Figure 61. Boxplot charts depicting the distribution of ASV abundance in the different categories of wine tasting attendance for each group (mean: 1-3 times a week – 17, one time per day – 17, one time per week – 17); p=0.82

The variable age and the remaining categories of taste were analysed and presented in Supplementary Figures 29 to 34. The category of age was divided into 3 categories by percentiles. The category 3 presented the higher and lowest value of Shannon index, and the category 2 showed the highest and lowest value of ASV abundance

4.4.5.1. Beta Diversity

Bray Curtis dissimilarity distances were calculated to measure the differences between individuals, to consider phylogeny it was calculated the unweighted frac with PERMANOVA. In the Figures 63A and 63B are represented the beta diversity values between samples from the two groups, and the distances to control and wine taster group respectively. According to the beta diversity values, there is no differentiation in the microbiome composition between the two groups (Pseudo-F = 0.649, p=0.891).

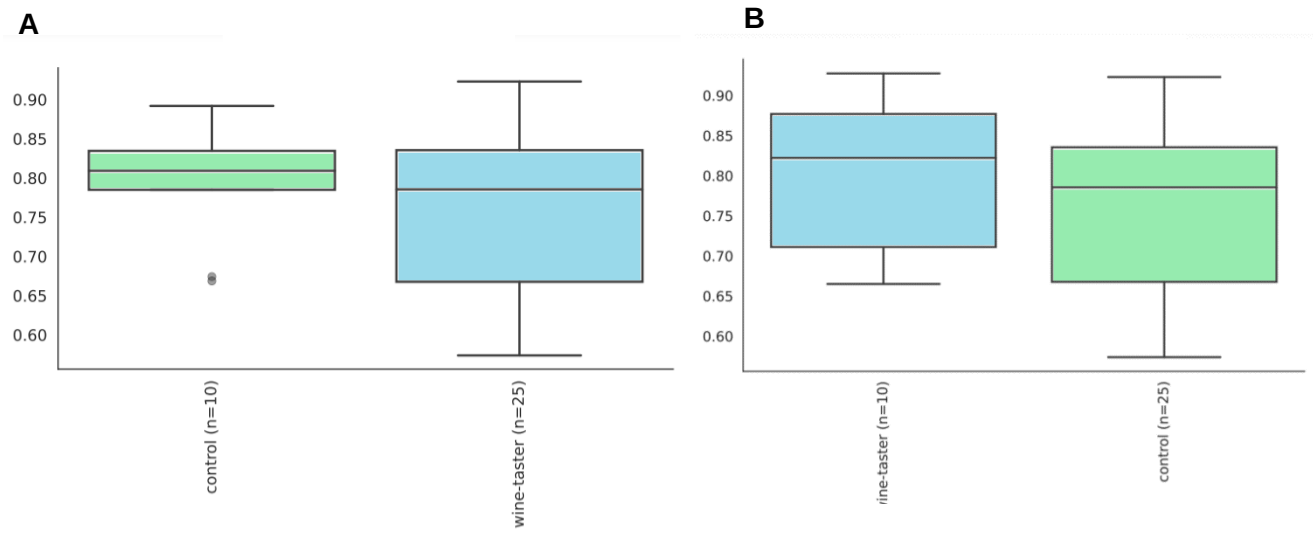

Figure 62. Unweighted frac values within each of the two groups and between them. (A) distance to control group (B) distance to wine taster group. Control samples in green color and wine tasters in blue color

The beta diversity distances between samples belonging to the same gender were different to the distances obtain when comparing samples of different gender (Pseudo-F = 1.11, p= 0.404). In other words, there is no difference in the microbiome composition when comparing the variable gender (Figure 64).

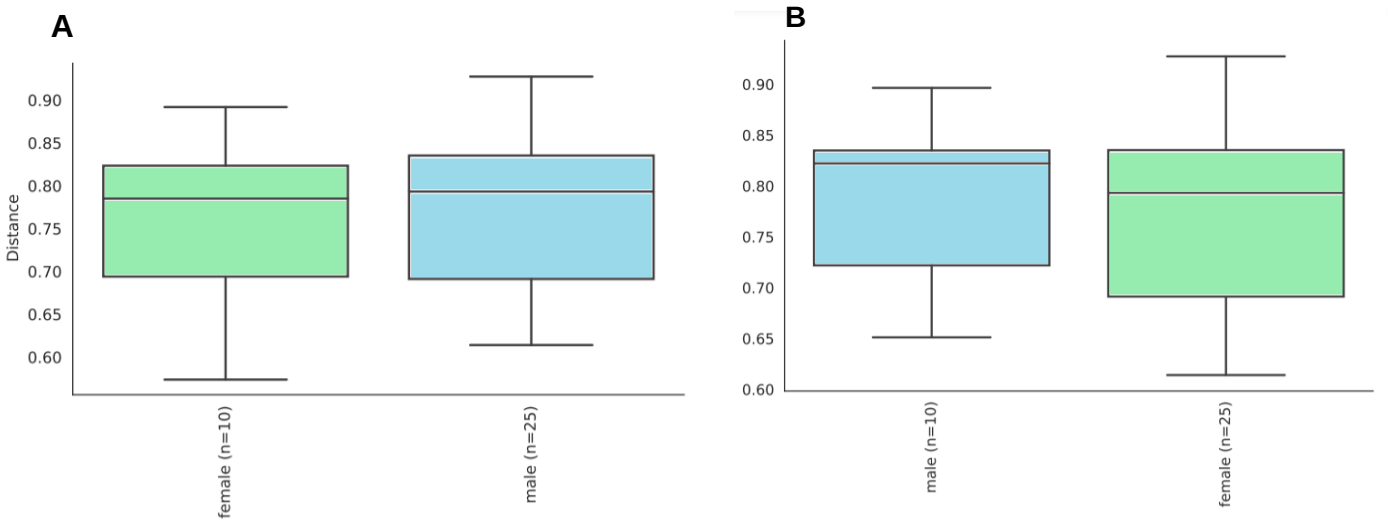

Figure 63. Unweighted frac values within each of the two groups and between them. (A) distance to female (B) distance to male, female samples in green color and males in blue color

The principal coordinates analyses (PCoA) plot from the Bray Curtis dissimilarity matrix was performed for a better understand if existed a taxonomic structure in the samples analysed. The plot revealed a major aggregation between axis 2 and 3, with most of the samples (Figure 65). Only three female samples formed outside group, two belonging to control and one to wine tasters' group.

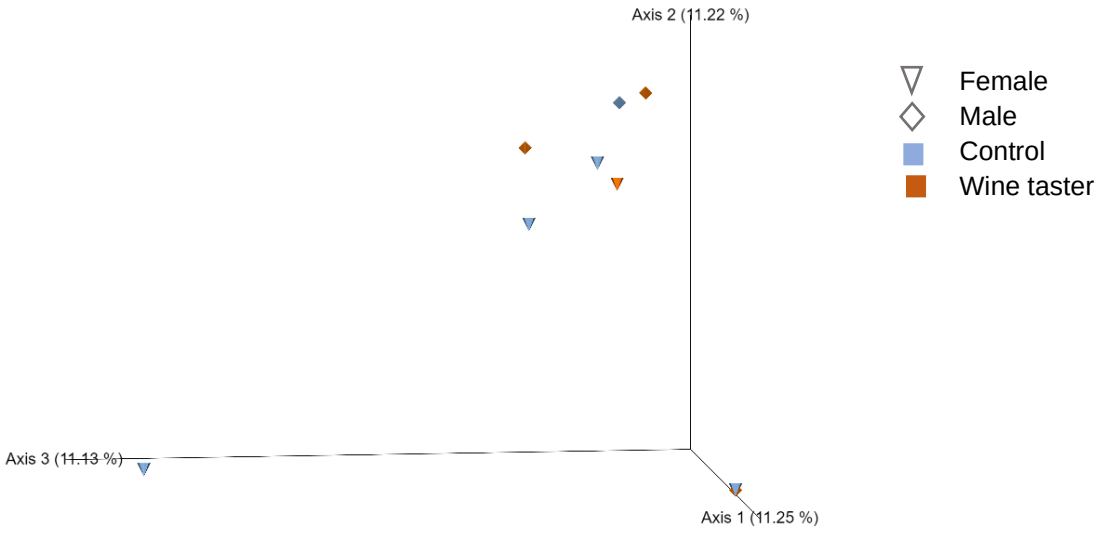

Figure 64. PCoA plot from Bray Curtis dissimilarity matrix. Percentage of the total variance represented by each axis is provided within parenthesis next to the label of the axis. Each shape represents one sample.

4.4.6. Characterization of the Diet and Lifestyle

The variables evaluated for this characterization were the habits of consumption of the different foods in each category of taste (sweet, sour, astringent, and bitter), the use of condiments, consumption of alcohol, and smoking habits. Only for the wine tasters' groups was considered the frequency of attendance in wine tastings, career length, and care before tasting events. For each of those variables mentioned before, their distributions between the two groups (control and wine tasters) were compared through a crosstab with a Chi-square of Pearson, with a 5% significance level. Considering the "Frequency of consumption of foods sweet-taste related", all the individuals of both groups revealed the same frequency of consumption, demonstrating no difference between groups (Supplementary Table 19). The frequency of consumption of foods representing the bitter, sour and astringent was not significantly different between groups with the correspondent p values of 0.097, 0.198, and 0.150. The tables with the counts for each group are represented in Supplementary Table 20, 21 and 22.

When comparing the use of condiments between the two groups, only the use of sugar (p=0.010) was significantly different between groups, the other condiments are represented in Supplementary table 23. "Smoking habits" were also compared having no significant difference between groups (p=1.00) (Supplementary Table 24).

The same variables were compared between gender, where the "Frequency of sweet-related foods" obtained the same frequency for all the individuals, not being possible to perform the test (Supplementary Table 25). Concerning the "bitter and astringent-related foods", non-significant difference between genders was found (Supplementary Table 26 and 27). The "Frequency of consumption of sour-related foods", obtained a significant difference (p=0.010) (Supplementary Table 28), having a higher frequency of consumption in females. The "Higher frequency of consumption of vinegar" (p=0.010) and "pepper" (p=0.038) was also significantly different between gender. The remaining variables were not significantly different (Supplementary Table 29).

The frequency of tasting different types of wine, the "Frequency of attendance to tastings", and "Higher sensibility to taste" were also compared between career length, but because it was the same between all the individuals, this test was impossible to perform. Instead, the same variables were compared between gender, but neither was significantly different (Supplementary Table 30,31,32). The "Frequency of attendance to wine tastings" was also tested but not demonstrated a significative difference between genders (Supplementary Table 33). Regarding "Higher sensibility to taste", we can say

that females considered to have “Equal sensibility for all tastes” (Supplementary Table 34).

4.4.7. Correlation between Diet, Lifestyle, and Nasal Microbiome

Using the variables related to diet and lifestyle mentioned in the previous topic, we investigated if whether there was a significant association between these variables and the nasal microbiome. For this purpose, the Spearman correlation was used with the different variables and the identified taxa up to the genus level from each database.

Greengenes database: With the variable “Smoking habits” a negative correlation was presented with the taxa Neisseraceae (p=0.035), *N09* (p=0.035), and *Actinomycetales* (p=<0.001).

Silva database: Neisseraceae demonstrated a negative correlation with the variable “Smoking habits” (p=0.035).

RDP database: Halobacteria showed a positive correlation with the “Frequency of consumption of bitter-related foods” (p=0.035). Also presented a negative correlation with the variable “Higher sensibility to sweet and sour taste” (p=0.007). Actinobacteria presented a positive correlation with the variable “Higher sensibility to bitter taste” (p=0.044), and with “Higher frequency of tastings with sparkling wine” (p=0.044).

5. DISCUSSION

This study aimed to characterize the tongue dorsum and nasal microbiota in wine tasters from Portugal using the 16S rRNA sequencing method. Variables as diet, oral hygiene and lifestyle habits were taken into account as they may influence both microbiomes. The wine tasters' group was compared to a non-wine taster group (control group). For this comparison, the taxonomic alignment was performed with four different databases (Greengenes, SILVA, RDP, and HOMD) to obtain the best percentage of taxonomy assigned. Geengenes last actualization was in 2013, SILVA was actualized in 2019, RDP (Ribosomal Database Project) was updated in 2016 and HOMD (the oral microbiome database) was actualized in 2020. According to previous studies, "unclassified" taxa is due to the unavailability of taxonomy for the sequence at that taxonomic level in respective databases. This could indicate the incompleteness of databases at various taxonomic levels and, to investigate such bias, we decided to utilize different databases. Dixit *et al*. (2021) used SILVA, Greengenes and EzTaxon databases for identifying 16S rRNA gene sequences obtained from RDP official website and found that the database with the best performance was SILVA with an identity threshold of 99%. Our data indicates the contrary, with Greengenes performing as the database with the higher number of identified taxa in both types of samples. Surprisingly HOMD was not the best database identifying the dorsum tongue microbiome since it is a specific oral database and, also it was the database updated more recently. The worse performance for buccal samples was RDP meanwhile, for nasal samples was HOMD. This result was also unexpected considering previous works examining nostril samples, where HOMD obtained the best results compared with SILVA128, RDP16, NCBI 16S and Greengenes (Escapa *et al.*, 2018). Our results are probably related to the small number of samples from the nostril along with the paired-end configuration. Also, the nasal microbiome is less studied and therefore these microorganisms remain undescribed. Nevertheless, there was a high percentage of taxa that was not possible to identify in both microbiomes. Our target sequence was the V3-V4 region of the 16S rRNA, with a size of 460 bp. The sequencing strategy was 2x150 configuration and thus, the sequencing of the V3-V4 region was incomplete. This feature may be responsible for the low identification results. It is necessary to use a 2x250 paired-end configuration to increase the power of identification.

Considering the oral and nasal microbiome, the control group presented a higher number of taxa when compared to the wine tasters' group in both cases. This result is consistent with all databases analyzed.

5.1. Tongue Dorsum Microbiome

The control group showed a higher number of taxa than both the wine taster groups. Regarding the samples' pair from the wine taster group, the sample collected before the wine tasting, in general, presented a higher number of taxa than the samples collected after the wine tasting.

Over taxonomic assignment, at the phylum level, all groups were dominated by Firmicutes, Proteobacteria, and Actinobacteria in three databases (Greengenes, SILVA and RDP). For HOMD the most dominant phyla were *Firmicutes*, *Fusobacteria,* and *Bacteroidetes*. Previous studies also reported the same taxa in the oral cavity (Dwivedi *et al.*, 2019; Neyraud & Morzel, 2019; Zaura *et al.*, 2009), with *Firmicutes* and *Bacteroidetes* more related to the microbiome of the tongue dorsum.

At the class level, the most abundant taxa was the Actinobacteria, except in the HOMD database which was Bacilli. The main differences between groups that are present in all databases are the higher abundance of Bacilli in the prov2 group, Firmicutes with higher abundance in both wine tasters' groups, and higher abundance of Bacteroidia in the control group. A higher presence of Actinobacteria relates to less sensitivity to taste (Cattaneo *et al.*, 2019). Although it was expected that the control group showed a higher abundance of this taxa, it was the wine tasters' group that displayed a higher percentage of this bacteria class.

At the genus level, the most dominant taxa identified by the Greengenes and SILVA databases was *Actinomyces*. The main differences between all databases were the higher abundance of *Streptococcus* in the prov2 group and the higher abundance of *Veillonella* in both wine tasters' groups. No specific studies on the wine tasters' microbiome were assessed, but investigations where the study group were individuals with a higher sensibility to taste, namely sensibility to PROP, the genera *Actinomyces*, *Oribacterium*, *Solobacterium*, *Catonella,* and *Campylobacter* were more abundant. This was not in accordance with our results, Campylobacter was present in prov1 group and control group, and *Catonella* and *Oribacterium* were only found in the control group (Cattaneo *et al.*, 2019). However, the phyla *Firmicutes* was more abundant in the wine tasters' groups, but in different genera from those mentioned in the study, such as *Gemella*, *Catonella* and *Veillonella*, possibly connecting these genera also with a higher sensitivity to taste. Unfortunately, it was not possible to accomplish the species-level identification for all taxa.

Oral bacteria can spread through the body and are often associated with several systemic diseases that are not limited to the oral cavity (Wade, 2013; Willis & Gabaldón,

2020). Some disease-related, such as *Campylobacter,* often associated with digestive infections, were found in some individuals, one of the prov1 group and four individuals of the control group. According to Sampaio-Maia *et al.* (2016), this taxon is also considered an oral periopathogen stimulated by stress. Besides, some genera are related to endocarditis and osteomyelitis such as *Gemella*, only present in the control group. The other disease-related taxa are associated with gingivitis and periodontitis. Gingivitis is the most common bacterial disease, the primary colonizers tend to be *Actinomyces*, *Fusobacterium,* and *Treponema* species (Wade, 2013), which were also observed in our samples. Regarding periodontitis, tobacco smoking is a strong environmental factor, and the taxa associated with this disease are *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (Wade, 2013). Some of our identifications were not possible to attain the species level. Regarding these phyla only *Treponema* and *Porphyromonas* were present in our samples. *Treponema* was found exclusively in control samples. In addition, taxa related to the fermentation processes and the production of lactic acid involved in food digestion, such as *Carnobacteriaceae*, *Lactobacillales*, *Carnobacteriaceae* and *Actetobacteraceae,* were observed.

The genus *Streptococcus* had a significant presence in the prov2 group compared with the other two groups, identified by all databases. *Atopobium*, *Lachnoanaerobaculum*, *Schaalia* and *Ruminococcaceae* were only found in prov2 group. According to a previous study, *Ruminococcaceae* is associated with a higher energy, fat and protein intake, and *Lachnoanaerobaculum* with a higher sensibility to sour taste (Cattaneo *et al.*, 2019).

The dorsum tongue microbiome of the Portuguese population was never studied in deep. Up to date, only one study investigated the oral microbiome of the Portuguese population (Almeida-Santos *et al.*, 2021). But this study used saliva samples which microbiome is partially shared with that of all diverse sites of the buccal cavity due to contact and found that the most abundant phyla were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria, which is similar to our findings. At the genus level, Almeida-Santos *et al.* (2021) identified that the most abundant were *Streptococcus*, *Prevotella* and *Neisseria*. In our study were identified *Veillonella* and *Streptococcus*, adding also *Actinomyces* at the findings of the previous work.

Regarding alpha diversity values, both ASV abundance and Shannon Index were not significantly different among the three groups. But it was possible to observe that the ASVs abundance was higher in the control group, and the Shannon index was higher in the prov1 group. ASVs abundance expresses the microorganisms' richness, and the Shannon index indicates the evenness of the samples. Our results demonstrate that the control group had a higher richness of taxa but a less even distribution. The prov1 group

had a lower taxa richness than the control group but with a more even distribution. The prov2 had less richness of taxa and less even distribution compared with control and prov1 groups.

When comparing by gender, the values of ASV abundance and Shannon index were not significantly different. But our results showed an increase of diversity in women (16.8) compared with men (9.8) according to the ASVs abundance in the control group.

In the Shannon index, observed an increase in women in the control group (2.7) and prov2 (3.2) comparing to men (control: 2.1, prov2: 2,2). On the other hand, men of the prov1 group had higher values of diversity (women: 2.5, men: 3.1).

When considering the oral hygiene variables, there was no significant difference when comparing groups of frequency of tooth brushing or use of mouthwash, just an increase of diversity in the tongue dorsum microbiome was observed in the non-users of mouthwash. Previous studies about the effect of mouthwash in oral plaque indicate that it presents antimicrobial properties, reducing the oral plaque. The effectiveness of the mouthwash depends on the components of the mouthwash. Listerine is one of the most popular phenolic mouthwash, presenting moderate effects on plaque inhibitory and anti-gingivitis (Farook & Said, 2018). In our subjects, the frequency of the use mouthwash was relatively high and, therefore, If tooth cleaning is regular, the disease-related bacteria would be kept in an immature state and in small amounts, creating a healthy environment (Wade, 2013).

Concerning the variables related to diet and lifestyle, only in the variable “Consumption of foods related to sweet-taste” was verified a significant difference when comparing the different categories of consumption. So, our results indicate that there is a difference in the richness of the microbiome of subjects with different levels of consumption of sweets. The variable “Alcohol consumption” was compared between the control group and the two groups of wine tasters’ and among all the subjects obtaining no significant difference between groups or all subjects. The same result was obtained when comparing the variable “Frequency of attendance to wine tastings” and “Career length of wine taster” between the two groups of wine tasters. For the other variables, a clear pattern of their influence in the tongue dorsum microbiome was not found.

The beta diversity showed a significant difference in the dorse tongue microbiome among groups, the distances within a group are different from the distance between groups. The composition of the oral microbiome is different between the control group, prov1 e prov2 groups. Therefore, there was a difference in microbial composition between groups. This result was not reflected in the heatmap where samples from the three groups were

present in the two main clusters. However, with PCoA it is possible to observe outside groups from the prov1 and control group, indicating a difference between groups. Regarding richness, as mentioned before, ASV abundance and Shannon Index were not significantly different between groups. The trend displayed by these analyses support the findings of beta diversity analysis since the results of the ASV abundance showed that prov2 displayed small richness values. In fact, this was expected as it is widely known the bactericidal properties of the alcohol. However, when comparing the group control+prov1 with prov2 group with the PERMANOVA test there was no significatively difference between groups, which could be biased by the different sample sizes (control+prov1 – 35; prov2 – 18 individuals). The consumption of alcohol is not significantly different between groups, although when comparing the diversity richness of the groups (Shannon index) the Kruskal Wallis test indicated that within the control group, there was a significant difference in the richness of the individuals consuming alcohol more than two times a week and less than one time per month or never. Also, significant differences were found within the group prov1+prov2 in individuals consuming alcohol, ingesting an average of one time per week and more than two times a week. These results showed that alcohol consumption affects the richness of the oral microbiome, which was highlighted by the results of the beta diversity estimates between the control and wine tasters' groups (prov1+prov2), which showed a significant difference in the oral microbiome composition between groups. These results are in agreement with the hypothesis made by Requena *et al.* (2010), that moderate consumption of wine can modulate the oral microbiota. Finally, it is important to state that our volunteer's' enquiry did not ask to discriminate the type of alcohol beverage, and we recommend its inclusion in future studies.

As mentioned before, the beta diversity analysis was also performed comparing the control group with prov1+prov2 group (wine tasters), with a significantly difference between groups, stating the fact that the control samples and the wine taster samples are more different from each other than prov1 or control and prov2 samples. This information can indicate that the tongue microbiome of wine tasters is different from the non-wine tasters due to their professional activities.

As other variables can influence microbiome composition and abundance and for this reason were a target of the present study. Understanding the association of the tongue dorsum microbiome with factors as diet, lifestyle, and oral hygiene habits is crucial. According to our results, certain variables such as career length is correlated with the genera Pasteurellaceae. *Porphymonas*, *Actinobacillus*, *Weeksellaceae*, *Oribacterium* and *Stomatobaculum* presented a correlation with "Frequency of consumption of foods

from sweet taste". Kato *et al.* (2017), reported that higher levels of carbohydrates intake in the diet showed an increase in the abundance of *Lactobacilli* and *Streptococcus mutans* in the oral cavity. This affirmation is not consistent with our results, probably due to the subjectivity of the personal data about dietary and lifestyle habits.

Lactobacillales presented a correlation with the variable "Higher sensibility to sweet-taste" in two databases (Greengenes and SILVA); Gemellaceae were correlated with "Higher frequency of consumption of paprika" and "Higher frequency of consumption of bitter taste"; *Haemophilus* were correlated with "Higher sensibility to sour and bitter taste" and "Higher sensibility to sweet and bitter taste". Although a recent study found alterations, particularly due to the higher abundance of Clostridiales and Bacteroidetes in the oral microbiota from individuals presenting a higher sensitivity to salty and sour taste (Cattaneo *et al.*, 2019), our results do not support those findings regarding the sour taste. As we did not evaluate the variable related to salty taste, and in our variables the bitter taste is present in both ("Higher sensibility to sour and bitter taste" and "Higher sensibility to sweet and bitter taste"), it is possible that the identified taxa may be more related with a higher sensibility to bitter taste.

5.2. Nasal Microbiome

The obtained results showed that the control group displayed a higher number of taxa than the wine taster group, which is in line with what we observed for dorse tongue microbiome. Also, it is important to mention that the lack of previous studies on the nostril microbiome of Portuguese individuals prevent us to compare our data.

In terms of taxonomic assignment, at the phylum level, both groups were dominated by Proteobacteria, Actinobacteria, and Firmicutes. This outcome was similar for all databases. At the class level, the dominant taxon between all groups and databases was the Actinobacteria. The main difference between groups was the higher abundance of Alphaproteobacteria in the control group and Clostridia in the wine tasters' group. At the genus level, *Corynebacterium* was the dominant genus between all databases except in the RDP database, which only identified Actinobacteria in both groups. The main differences between groups demonstrated by Greengenes and SILVA was a higher abundance of *Brachybacterium* in the control group. The wine tasters' group had a higher abundance of N09 and *Peptoniphilus* in the Greengenes database. In the RDP database, the main difference was the presence of *Caldanaerobacter* and Halobacteria in the control group. Previous studies on the nasal microbiome demonstrated that the phyla such as Firmicutes and Actinobacteria and genera such as *Corynebacterium,*

Propionibacterium, and *Staphylococcus* are “normal” residents of the nostrils (Lemon *et al.*, 2010), which is in line with our results. Comamonadaceae was only identified in wine tasters’ group, and it was associated in previous investigations with reduced odor threshold (Koskinen *et al.*, 2018). However, this taxa was only found in one subject (47N) and in a low percentage (0.001%).

Regarding the alpha-diversity estimates, both the ASV abundance and the Shannon index were not significantly different between groups. The Shannon index between groups was similar, but we can see a higher ASV abundance in the control group (22.2) compared with wine tasters' (16.8). About the variable gender, there was also no significant difference in the ASV abundance and Shannon index. The major difference was exhibited when comparing the gender divided by groups, with a lower value of ASV abundance displayed by the males of the control group. When comparing the other variables related to diet, lifestyle, and oral hygiene habits no significant difference was observed. The beta-diversity estimates showed no differentiation in the nasal microbiome composition between both groups or gender, which was supported by the heatmap and the PCoA where there is not a defined cluster formed in either gender or groups samples.

Finally, the last part of the study was to test whether there was a correlation between the evaluated values and the composition of the nasal microbiome. According to the comparison of variables with Greenegenes and SILVA databases, the variable “Smoking habits” was correlated to Neisseraceae, *Actinomycetales*, and *N09*. This is quite contrasting with a study of smokeless tobacco (Shammah) users and non-smokers from Saudi Arabia, where the taxa *Actinomyces* and *Streptococcus* were correlated with smokeless tobacco consumption (Halboub *et al.*, 2020). When using the RDP database taxonomic identification, an association between Halobacteria and the variables “Frequency of consumption of bitter-related foods” and “Higher sensibility to sweet and sour taste” was found. Also from this database, Actinobacteria was correlated with “Higher sensibility to bitter-taste” and “Higher frequency of tastings with sparkling wine”. Finally, we did not find any disease-related bacteria, but some environmental bacteria, such as Bradyrhizobiaceae and Caulobacteraceae were observed.

Regarding taxa identification in the nasal microbiome, not only the configuration of sequencing might be responsible for the lower taxonomical identification power, but also, the available databases have few information (sequence references) from nasal microbiome, e.g., in our nasal data 97% of taxa were Unassigned. Another finding of this study was higher number of taxa displayed by the tongue dorsum microbiome and nostril microbiome composition, the first showed a higher number of taxa. Nostril samples

retained more reads than dorse tongue samples after rarefaction, but with a higher Unassigned percentage. Another fact that could influence the taxa counts is the small sample size of the Nasal microbiome.

At the phylum level, Bacteroidetes, Fusobacteria, SR1, Planctomycetes and Spirochaetes are only present in the tongue dorsum samples, and Firmicutes, Actinobacteria, Proteobacteria and OD1 are shared between oral and nasal samples. At the class level, the taxa Bacteroidia, Coriobacteria, Epsilonproteobacteria and Fusobacteria are only present in samples from the tongue dorsum. The *Actinobacteria*, Alphaproteobacteria, Bacilli, Betaproteobacteria, Clostridia and OD1 were the taxa shared between the oral and nasal microbiome.

At the genus level, Acetobacteraceae*, Brachybacterium,* Bradyrhizobiaceae*,* Comamonadaceae*,* Instransporangiaceae *and Peptinophilus, Staphylococcus, Bifidobacterium,* Xanthobacteraceae*, Beijerinckia* and *Caldanaerobacter* are the most abundant taxa only present in nostril samples. *Bacteroidales, Haemophilus,* Pasteurellaceae*, Veillonella, Streptococcus, Lactobacillus, Megasphaera, Alloprevotella, Aggregatibacter, Granulicatella,* Lachnospiraceae*, Rothia, Porphyromonas* and Ruminococcaceae are the most abundant taxa which were only observed in the tongue dorsum samples.

The present work was the first study that integrate the analysis of the tongue dorsum and the nostril microbiome from a group of wine tasters, and the first on the tongue dorsum and the nasal microbiome of the Portuguese population. Therefore, the generalizability of the present study remains to be defined. Another important aspect of the obtained results is the fact that they were obtain from a part and not the whole region v3-v4 sequence. As a first of the kind, there are some aspects that need to be improved for a larger taxonomic resolution that will permit a better understanding of the association between microbiome composition and taste perception.

6. CONCLUSIONS

A basic but important general conclusion of this work, to consider in future studies with the same objectives, is the indication that the Greengenes database was the one providing better results for both nasal and tongue microbiomes.

6.1. Tongue dorsum microbiome

- The control group had a larger number of identified taxa (48) than the wine tasters' group (38), 56% of all identified taxa were shared between groups.
- Alpha diversity analysis: the variable the sweet category obtained a significantly difference between groups, indicating that the richness of all the subjects was significantly different according to different categories of consumption of foods sweet-related.
- Alpha diversity analysis: Some differences at the genus and class level between groups were also found between groups, and when comparing different lengths of wine taster career.
- Differences between the groups (Control, Prov1 and Prov2) where found through beta diversity analysis. The composition of the oral microbiome is significantly different between the control group and the other two groups and can be related to wine taster professional activities.
- A difference between genders was also found, indicating that the tongue dorsum microbiome composition of females and males is diverse.
- Correlation between some lifestyle and diet variables and the composition of the microbiome were found, such as the use of mouthwash, and the consumption of some taste-related foods and condiments, showing these variables as influencing factors.

6.2. Nasal Microbiome

- The control group obtained a higher number of identified taxa (19) than the wine tasters' group (12). 40% of all identified taxa were shared between groups.
- Alpha diversity analysis and Beta diversity measures do not indicate differences in the composition of the nasal microbiome between the two groups.
- Smoking habits and consumption of some taste-related foods, showed to be influencing factors in the composition of the nasal microbiome.

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8. SUPPLEMENTARY MATERIAL

Index of Tables

Index of Figures

Supplementary document 1 – Informed Consent

Termo de Consentimento Informado

"Caracterização do microbioma do dorso da língua de provadores de vinho"

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Enquadramento: Esta investigação é realizada no âmbito de uma Tese de Dissertação do Mestrado de Ciências do Consumo e Nutrição, na Faculdade de Ciências do Porto. Na qual integra as áreas de estudo como genética, microbiologia e nutrição. As novas técnicas de sequenciação de ADN modernas vieram a possibilitar a caracterização dos microrganismos (por exemplo, bactérias, fungos, leveduras) que povoam um determinado ambiente, ao que vulgarmente se designa por **microbioma**. Dados recentes têm vindo a mostrar que os microbiomas de certos órgãos ou regiões corporais (por exemplo, boca, intestinos, ouvidos, nariz) têm não só uma função de proteção ambiental, como também modulam o desempenho destes órgãos. No caso do **microbioma oral**, este tem uma constituição diversificada devido ao contínuo contacto que tem com o ambiente externo. O objetivo deste estudo prende-se na caracterização do dorso da língua de provadores de vinho, a partir da recolha de uma amostra do dorso da língua. Assim, será possível testar se estes são influenciados pela frequência das provas de vinho e, uma vez que as papilas gustativas se encontram localizadas no dorso deste órgão, testar se existe alguma relação entre o microbioma e a capacidade sensorial dos provadores. Para tal, serão recolhidas amostras de um conjunto de provadores de vinho e comparadas a um grupo controlo, constituído por uma amostra de pessoas que não têm qualquer relação com esta atividade, e distribuição etária semelhante à do grupo de referência.

Explicação do estudo: A passagem de uma zaragatoa no dorso da língua dos participantes, recolherá uma amostra do microbioma deste órgão. Esta recolha é voluntária e pode ser recolhida pelo próprio voluntário, em completa segurança, não envolvendo terceiros. Antes da recolha será dado a ler este texto e após aceitação será entregue um manual explicativo com todos os passos para auxiliar o processo de recolha e duas zaragatoas. Para os provadores de vinho a primeira recolha deverá ser feita nos instantes que antecedem a primeira prova do dia, e a segunda nos instantes seguintes à última prova do dia (caso estas sejam seguidas, caso não sejam, devem apenas refletir um bloco de provas). Será também pedido o preenchimento de um questionário realizado a ambos os grupos, com o objetivo de caracterizar os hábitos alimentares, estilo de vida e, no caso dos provadores fatores mais relacionados com a sua atividade e hábitos de preparação

para provas de vinho. O preenchimento deste questionário poderá ser via online ou presencial na altura da recolha da amostra, ficará ao critério do participante. Por último, propõe-se a realização de um teste de densidade de papilas gustativas, sendo este é um teste não obrigatório ficando a critério de cada voluntário. Caso haja concordância do voluntário, será tirada uma fotografia da língua para contagem posterior das papilas gustativas.

Confidencialidade e anonimato: Os dados recolhidos serão de uso exclusivo para o estudo, a informação será codificada e protegida. Os resultados do estudo podem ser partilhados individualmente, apenas e só, com os participantes caso estes manifestem interesse, indicando a sua vontade aquando do preenchimento do questionário, bastando para isso, fornecer o seu email pessoal. O acesso aos dados de cada um dos participantes é apenas e só revelado individualmente a cada participante, estando absolutamente vedado às instituições a cujos participantes possam estar vinculados, mesmo com autorização do participante.

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela/s pessoa/s que acima assina/m. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. 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 pelo/a investigador/a.

Nome: _____
Assinatura: _____
Data: ___ /____/_____

_____ _____

(Albano Beja-Pereira) (Sofia Coimbra

Supplementary document 2 – Questionnaire

U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

U.PORTO
FACULDADE DE CIÊNCIAS DA NUTRIÇÃO E ALIMENTAÇÃO
UNIVERSIDADE DO PORTO

Questionário

Caracterização do microbioma do dorso da língua de provadores de vinho

Sou estudante do Mestrado em Ciências do Consumo e Nutrição, e no âmbito da minha tese de mestrado, que tem como objetivo compreender a influência da atividade de provador no microbioma oral, e as diferenças entre provadores de vinho e não provadores. Para tal venho solicitar a colaboração no preenchimento deste questionário, para uma melhor compreensão de fatores externos como estilo de vida, saúde oral e hábitos alimentares, que possam também estar na origem desta alteração da composição do microbioma oral.

Código interno: __________

☐ Ao assinalar cum uma cruz a caixa ao lado, declaro ter lido e compreendido a descrição do estudo apenso a este inquérito e por tal aceito responder ao inquérito e ceder uma amostra do dorso da língua voluntariamente.

Deseja receber os seus resultados individuais deste estudo?

☐ Sim email pessoal: _____@_____ ☐ Não

Quer participar na recolha de imagem das papilas gustativas com o fim de caracterizar a sua densidade, autorizando para tal que lhe seja tirada uma foto do seu dorso da língua?

☐ SIM

Secção I – Dados Sociodemográficos

1. Sexo: ☐ Feminino ☐ Masculino

2. Idade: _____

3. Localidade de Residência: _____

4. É provador de vinho? ☐ Sim ☐ Não

Se respondeu, **não** à pergunta anterior, por favor salte para a **secção 3** deste questionário.

Secção II – Hábitos Provadores de Vinho

1. Há quanto tempo possui a profissão de provador de vinho?

☐ Menos de 5 anos

☐ Entre 5 a 10 anos

☐ Entre 10 a 15 anos

☐ Mais de 15 anos

2. Com que frequência realiza provas de vinho?

☐ Menos de 1x p/mês

☐ 1-3 x p/mês

☐ 1 x p/semana

☐ 2-4x p/semana

☐ 5-6x p/semana

☐ 1x p/dia

☐ Mais de 1x p/dia

3. Há quanto tempo participou numa prova de vinho? _____

4. Quantas provas de vinho realizou nas últimas duas semanas? _____

5. Quantas amostras de vinho (aproximadamente) provou nas últimas duas semanas? _____

6. De acordo com a sua opinião pessoal, acha que é mais sensível a que sabor? (Escolha a opção(s) com que mais se identifica)

☐ Doce

☐ Amargo

☐ Ácido

☐ Sou sensível a todos por igual

7. Que tipo de vinhos prova com maior regularidade?

☐ Licorosos

☐ Tranquilos

☐ Espumantes

8. Com que frequência prova licorosos e número médio de vinhos por prova?

☐ Diária

☐ Semanal

☐ Bissemanal

☐ Mensal

9. Com que frequência prova tranquilos e número médio de vinhos por prova?

☐ Diária

☐ Semanal

☐ Bissemanal

☐ Mensal

10. Com que frequência prova espumante e número médio de vinhos por prova?

☐ Diária

☐ Semanal

☐ Bissemanal

☐ Mensal

11. Além do que consome durante as provas há algum destes tipos de vinho que consuma frequentemente (diariamente)?

☐ Sim ☐ Não

a) Se sim, Qual? _____

12. Nas 48 horas anteriores a uma prova tem cuidados especiais?

☐ Sim ☐ Não

13. Se respondeu sim à resposta anterior, responda às perguntas seguintes:

13.1. Para de fumar?

☐ Sim ☐ Não

13.1.1. Se para de fumar, costuma fazê-lo?

☐ 48 horas antes.

☐ 24 horas antes.

☐ 12 horas antes.

☐ Menos de 12 horas antes.

13.2. Costuma beber mais água que o habitual?

☐ Sim ☐ Não

13.2.1. Costuma neutralizar entre provas com água?

☐ Sim ☐ Não

13.3. Deixa de lavar os dentes?

☐ Sim ☐ Não

13.3.1. Quanto tempo antes, tem esse cuidado?

☐ 48 horas antes.

☐ 24 horas antes.

☐ 12 horas antes.

☐ Menos de 12 horas antes.

13.4. Evita comer comida muito condimentada?

☐ Sim ☐ Não

13.4.1. Quanto tempo antes, tem esse cuidado?

☐ 48 horas antes.

☐ 24 horas antes.

☐ 12 horas antes.

☐ Menos de 12 horas antes.

13.5. Evita beber vinho?

☐ Sim ☐ Não

13.5.1. E outras bebidas alcoólicas?

☐ Sim ☐ Não

13.5.2. Quanto tempo antes, tem esse cuidado?

☐ 48 horas antes.

☐ 24 horas antes.

☐ 12 horas antes.

☐ Menos de 12 horas antes.

13.6. Consome vinhos diferentes do que os da provar, com a finalidade de apurar os seus sentidos?

☐ Sim ☐ Não

13.7. Se tiver outro tipo de cuidados, mencione abaixo, dizendo qual e quanto tempo antes _____

Secção III – Saúde Oral e Estilo de Vida

1. É fumador?

☐ Sim ☐ Não

1.1. Quantos maços de tabaco consome por semana? _____

2. Com que frequência lava os dentes?

☐ Menos de 1 vez p/dia

☐ 1 vez p/dia

☐ 2 vezes p/dia

☐ Mais de 2 vezes p/dia

2.1. Qual marca da pasta de dentes utiliza? _____

2.2. Usa elixir bucal?

☐ Sim ☐ Não

Se sim, qual a marca? _____

3. Com que frequência vai ao dentista?

☐ Em média menos de uma vez por ano

☐ Pelo menos uma vez por ano

☐ 2 – 3 vezes ao ano

☐ Mensalmente

3.1. Costuma ter problemas de saúde oral?

☐ Sim ☐ Não

Se sim, quais? _____

3.2. Tem aftas frequentemente?

☐ Sim ☐ Não

3.3. Tomou antibióticos no último mês?

☐ Sim ☐ Não

4. Com que frequência consome bebidas alcoólicas? (**se for provador, considere o consumo fora de provas**)

☐ Nunca

☐ Menos de 1x p/mês

☐ 1-3 x p/mês

☐ 1x p/semana

☐ 2-4x p/semana

☐ 5-6x p/semana

☐ 1x p/dia

☐ Mais de 1x p/dia

Secção IV – Hábitos Alimentares

1. A partir da lista de alimentos apresentada abaixo, assinale a resposta mais adequada de acordo com a frequência com que consome.

PRODUTO ALIMENTAR	Nunca	Raramente	Às vezes	Frequentemente	Muito Frequentemente
AMARGOS					
Chocolate Negro					
Iogurtes Naturais					
Rúcula					
Café					
Nabo					
Aipo					
Toranja					
Espinafres					
Espargos					
Endivas					
Couve de Bruxelas					
DOCES					
Chocolate de leite/branco					
Baba de camelo					
Refrigerantes					
Gelados					
Leite Condensado					
Caramelo					
Figos					
Uvas					
Banana Madura					
ÁCIDOS					
Abacaxi					
Maracujá					
Lima					
Framboesa					
Cebola					
ADSTRINGENTES					
Maçã					
Banana verde					
Cenoura					
Dióspiro					
Gengibre					
Laranja					
Limão					

2. Quais os condimentos que utiliza com mais frequência e de forma pouco esporádica na sua alimentação. Selecione da lista abaixo pelo menos um condimento que faça parte da sua alimentação diária:

☐ Sal

☐ Açúcar

☐ Molho de Soja

☐ Mostarda

☐ Colorau

☐ Alho

☐ Limão

☐ Caril (e.g., mistura tika massala, corma, etc)

☐ Gengibre

☐ Pimenta

☐ Vinagre

☐ Malagueta

☐ Cominhos

☐ Cravo das Índias

☐ Noz moscada

☐ Cebola

☐ Canela

☐ Açafrão

☐ Ervas aromáticas (Oregãos /manjericão/ salva/tomilho/ salsa/coentros

Supplementary document 3 – Step-by-step guide

Caracterização do microbioma do dorso da língua de provadores de vinho

Manual de Colheita

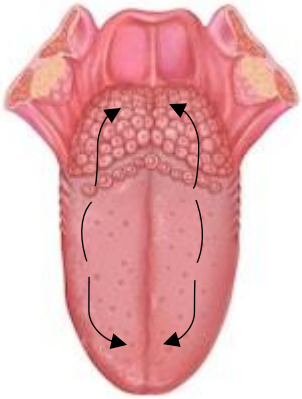
Este manual tem a função de auxiliar na recolha de amostra de placa bacteriana da zona dorsal da língua, siga os seguintes passos:

Nota importante: Só realizar a colheita se nas duas horas antecedentes não tiver comido, fumado, mastigado pastilhas ou ter lavado os dentes ou a boca com um elixir.

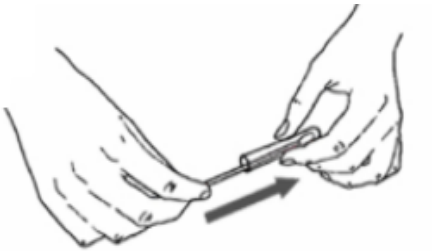
1º - Abrir a embalagem que contém a zaragatoa, de uma ponta à outra

2º - Retirar a zaragatoa tendo atenção para não tocar com a ponta branca em nenhum sítio

3º - Esfregar firmemente a ponta branca da zaragatoa contra a superfície dorsal da língua durante cerca de 60 segundos. Não tocar em mais nenhuma parte da boca que não seja o dorso da língua.

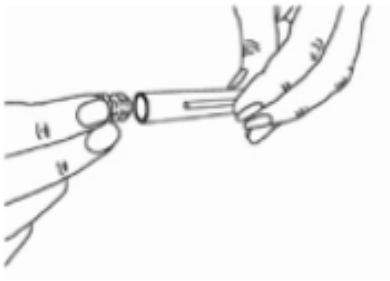


4º - Uma vez que as zonas sensoriais da língua estão espalhadas nas margens desta, passe a zaragatoa na direção das setas negras indicadas na figura ao lado.



5º - Abrir o tubo que contém o líquido para armazenar a zaragatoa, tendo cuidado para não tocar com a ponta branca na boca do tubo

6º - Faça pressão com a parte mais longa da zaragatoa contra a boca do tubo para que se solte a haste verde da ponta branca. A ponta branca deve ficar dentro do tubo e a haste verde é descartada.



7º - Feche o tubo firmemente

Fonte: Isohelix DNA Buccal Swabs

8º - Faça chegar o mais rapidamente possível a amostra à equipa responsável pela análise. Ver instruções em baixo.

Instruções de envio

- Após o fecho do tubo, colocar na embalagem fornecida para armazenamento;

- Por fim, entregar a uma das responsáveis presentes, por exemplo a Sofia Coimbra;

<u>Notas</u>

- Na embalagem encontra-se o código interno necessário para o preenchimento do questionário

- No final do código interno encontra-se um A e D, que indica que a amostra deve ser recolhida A – antes de uma prova e D- depois de uma prova (APENAS PROVADORES)

Oral Microbiome

***Supplementary Table 1* -** Crosstab and Chi-square of Pearson for variable "Consumption of sweet-taste related foods"

Group	Rarely	Sometimes	Frequently	Total
Prov1	1	14	0	15
Control	6	13	1	20
Prov2	3	13	1	17
Total	10	40	2	52
Chi-Square of Pearson	p= 0.392			

Supplementary Table 2 – *Crosstab and Chi-square of Pearson for variable "Consumption of sour-taste related foods"*

Group	Rarely	Sometimes	Frequently	Very Frequently	Total
Prov1	1	7	7	0	15
Control	2	9	8	1	20
Prov2	1	11	4	1	17
Total	4	27	19	2	52
Chi-Square of Pearson	p= 0.792				

Supplementary Table 3 - *Crosstab and Chi-square of Pearson for variable "Consumption of astringent-taste related foods"*

Group	Sometimes	Frequently	Very Frequently	Total
Prov1	7	6	2	15
Control	9	9	2	20
Prov2	8	9	0	17
Total	24	24	4	52
Chi-Square of Pearson	p= 0.667			

Supplementary Table 4 - *Crosstab and Chi-square of Pearson for variable.*

Group	Rarely	Sometimes	Frequently	Total
Prov1	4	9	2	15
Control	6	12	2	20
Prov2	3	11	3	17
Total	13	32	7	52
Chi-Square of Pearson	p= 0.906			

Supplementary Table 5 *- Crosstab and Chi-square of Pearson for variable "Regular consumption of condiments"*

		Groups				
Condiments		Prov1	Control	Prov2	Total	Chi-Square of Pearson
Salt	Yes	14	16	16	46	p=0.319
	No	1	4	1	6	
Sugar	Yes	4	3	5	12	p= 0.541
	No	11	17	12	40	
Garlic	Yes	13	13	16	42	p= 0.064
	No	3	7	1	10	
Lemon	Yes	9	8	5	22	p= 0.210
	No	6	12	12	30	
Curry	Yes	0	1	1	2	p= 0.650
	No	15	19	16	50	
Ginger	Yes	3	0	3	6	p= 0.118
	No	12	20	14	46	
Pepper	Yes	6	7	8	21	p= 0.757
	No	9	13	9	31	
Vinegar	Yes	5	11	4	20	p= 0.130
	No	10	9	13	32	
Chilli pepper	Yes	3	4	4	11	p=0.958
	No	12	16	13	41	
Cumin	Yes	0	0	1	1	p=0.350
	No	15	20	16	51	
Cloves	Yes	0	0	0	0	
	No	15	20	17	52	
Nutmeg	Yes	0	2	1	3	p=0.454
	No	15	18	16	49	
Cinnamon	Yes	1	1	3	5	p= 0.386
	No	14	19	14	47	
Turmeric	Yes	0	0	1	1	p=0.350
	No	15	20	16	51	
Herbs	Yes	7	10	12	29	p=0.319
	No	8	10	5	23	
Soy Sauce	Yes	3	0	3	6	p=0.118
	No	12	20	14	46	
Mustard	Yes	4	1	2	7	p=0.172
	No	11	19	15	45	
Paprika	Yes	0	3	0	3	p=0.078
	No	15	17	17	49	

Supplementary Table 6 *– Crosstab and Chi-square of Pearson for variable "Frequency of teeth brushing"*

Group	Less than one time a day	Once a day	Twice a day	More than twice a day	Total
Prov1	0	0	10	5	15
Control	0	3	13	4	20
Prov2	1	1	10	5	17
Total	1	4	33	14	52
Chi-Square of Pearson	p= 0.495				

Supplementary Table 7 *- Crosstab and Chi-square of Pearson for variable "Frequency of attendance in the dentist"*

Group	Less than 1 time per year	Least one time per year	2-3 times per year	Total
Prov1	0	11	4	15
Control	4	14	2	20
Prov2	2	11	4	17
Total	6	36	10	52
Chi-Square of Pearson	p= 0.338			

Supplementary Table 8 *- Crosstab and Chi-square of Pearson for variable "Mouthwash use"*

Group	Yes	No	Total
Prov1	6	9	15
Control	7	13	20
Prov2	5	12	17
Total	18	34	52
Chi-Square of Pearson	p= 0.820		

Supplementary Table 9 *- Crosstab and Chi-square of Pearson for variable "Smoking Habits"*

Group	Smoker	Non-smoker	Total
Prov1	4	11	15
Control	5	15	20
Prov2	4	13	17
Total	13	39	52
Chi-Square of Pearson	p= 0.979		

Supplementary Table 10 *- Crosstab and Chi-square of Pearson for variable "Consumption of bitter-taste related foods per gender"*

Group	Rarely	Sometimes	Frequently	Total
Female	5	10	3	18
Male	8	22	4	34
Total	13	32	7	52
Chi-Square of Pearson	p= 0.796			

Supplementary Table 11 *- Crosstab and Chi-square of Pearson for variable "Consumption of sweet-taste related foods per gender"*

Group	Rarely	Sometimes	Frequently	Total
Female	4	13	1	18
Male	6	27	1	34
Total	10	40	2	52
Chi-Square of Pearson	p= 0.812			

Supplementary Table 12 *- Crosstab and Chi-square of Pearson for variable "Consumption of sour-taste related foods per gender"*

Group	Rarely	Sometimes	Frequently	Very Frequently	Total
Female	1	8	7	2	18
Male	3	19	12	0	34
Total	4	27	19	2	52
Chi-Square of Pearson	p= 0.233				

Supplementary Table 13 *- Crosstab and Chi-square of Pearson for variable "Consumption of astringent-taste related foods per gender"*

Group	Sometimes	Frequently	Very Frequently	Total
Female	7	9	2	18
Male	17	15	2	34
Total	24	24	4	52
Chi-Square of Pearson	p= 0.663			

Supplementary Table 14 *- Crosstab and Chi-square of Pearson for variable "Frequency of wine tastings"*

Career Length	Less than 1 timer per month	One time per week	1-3 times per week	2-4 times per week	5-6 times per week	One time per day	Total
Less than 5 years	5	1	0	2	0	0	8
Between 5-10 years	2	2	2	0	0	0	6
Between 10-15 years	0	0	2	0	4	0	6
More than 15 years	0	1	1	3	1	6	12
Total	7	4	5	5	5	6	32
Chi-Square of Pearson	p= < 0.001						

Supplementary Table 15 *- Crosstab and Chi-square of Pearson for variable "Frequency of tasting liqueur wines"*

Career Length	Weekly	Biweekly	Monthly	Total
Less than 5 years	0	3	5	8
Between 5-10 years	0	2	4	6
Between 10-15 years	5	0	1	6
More than 15 years	0	2	10	12
Total	5	7	20	32
Chi-Square of Pearson	p= < 0.001			

Supplementary Table 16 *- Crosstab and Chi-square of Pearson for variable "Frequency of tasting calm wines"*

Career Length	Daily	Weekly	Biweekly	Monthly	Total
Less than 5 years	0	3	3	2	8
Between 5-10 years	0	6	0	0	6
Between 10-15 years	5	1	0	0	6
More than 15 years	8	3	0	1	12
Total	13	13	3	3	32
Chi-Square of Pearson		p= < 0.001			

Supplementary Table 17 *- Crosstab and Chi-square of Pearson for variable "Frequency of tasting sparkling wines"*

Career Length	Daily	Weekly	Biweekly	Monthly	Total
Less than 5 years	0	0	4	4	8
Between 5-10 years	0	2	0	4	6
Between 10-15 years	2	2	0	2	6
More than 15 years	7	3	0	2	12
Total	9	7	4	12	32
Chi-Square of Pearson		p= 0.002			

Supplementary Table 18 *- Crosstab and Chi-square of Pearson for variable "Higher sensibility for taste by groups"*

Group	Sweet	Bitter	Sour	Equally to all	Bitter+Sour	Sweet+Sour	Sweet+Bitter	Total
Prov1	1	4	1	6	0	2	1	15
Prov2	2	3	4	5	1	1	1	17
Total	3	7	5	11	1	3	2	32
Chi-Square of Pearson	p= 0.732							

Nasal Microbiome

Supplementary Table 19 *- Crosstab and Chi-square of Pearson for variable "Consumption of sweet-taste related foods"*

Group	Sometimes	Total
Wine taster	5	5
Control	5	5
Total	10	10
Chi-Square of Pearson		

Supplementary Table 20 *- Crosstab and Chi-square of Pearson for variable "Consumption of bitter-taste related foods"*

Group	Rarely	Sometimes	Frequently	Total
Wine taster	1	4	0	5
Control	0	2	3	5
Total	1	6	3	10
Chi-Square of Pearson	p= 0.097			

Supplementary Table 21 *- Crosstab and Chi-square of Pearson for variable "Consumption of sour-taste related foods"*

Group	Sometimes	Frequently	Total
Wine taster	4	1	5
Control	2	3	5
Total	6	4	10
Chi-Square of Pearson	p= 0.197		

Supplementary Table 22 *- Crosstab and Chi-square of Pearson for variable "Consumption of astringent-taste related foods"*

Group	Sometimes	Frequently	Very Frequently	Total
Wine taster	3	1	1	5
Control	1	4	0	5
Total	4	5	1	10
Chi-Square of Pearson	p= 0.150			

Supplementary Table 23 *- Crosstab and Chi-square of Pearson for variable "Regular consumption of condiments"*

		Groups			
Condiments		Wine tasters	Control	Total	Chi-Square of Pearson
Salt	Yes	5	4	9	p=0.202
	No	0	1	1	
Sugar	Yes	4	0	4	p= 0.010
	No	1	5	6	
Paprika	Yes	0	3	3	p=0.038
	No	5	2	7	
Garlic	Yes	4	5	9	p=0.292
	No	1	0	1	
Lemon	Yes	1	4	5	p= 0.058
	No	4	1	5	
Curry	Yes	0	0	0	
	No	5	5	10	
Ginger	Yes	1	4	5	p= 0.292
	No	0	5	5	
Pepper	Yes	1	2	3	p= 0.490
	No	4	3	7	
Vinegar	Yes	2	4	6	p= 0.197
	No	3	1	4	
Chili pepper	Yes	1	1	2	p=1.000
	No	4	4	8	
Cumin	Yes	0	1	1	p=0.292
	No	5	4	9	
Cloves	Yes	0	0	0	
	No	5	5	10	
Nutmeg	Yes	0	1	1	p=0.292
	No	5	4	9	
Onion	Yes	4	5	9	p=0.292
	No	1	0	1	
Cinnamon	Yes	1	2	3	p= 0.490
	No	4	3	7	
Turmeric	Yes	0	1	1	p=0.292
	No	5	4	9	
Herbs	Yes	3	3	6	p=1.000
	No	2	2	4	
Soy Sauce	Yes	0	0	0	
	No	5	5	10	
Mustard	Yes	0	0	0	
	No	5	5	10	

Supplementary Table 24 *- Crosstab and Chi-square of Pearson for variable "Smoking Habits"*

Group	Smoker	Non-smoker	Total
Wine Tasters	1	1	2
Control	4	4	8
Total	5	5	10
Chi-Square of Pearson	p= 1.000		

Supplementary Table 25 - *Crosstab and Chi-square of Pearson for variable "Consumption of sweet-taste related foods between genders"*

Gender	Sometimes	Total
Female	5	5
Male	5	5
Total	10	10
Chi-Square of Pearson		

Supplementary Table 26 *- Crosstab and Chi-square of Pearson for variable "Consumption of bitter-taste related foods between genders"*

Gender	Rarely	Sometimes	Frequently	Total
Female	0	3	2	5
Male	1	3	1	5
Total	1	6	3	10
Chi-Square of Pearson	p= 0.513			

Supplementary Table 27 *- Crosstab and Chi-square of Pearson for variable "Consumption of astringent-taste related foods between genders"*

Gender	Sometimes	Frequently	Very Frequently	Total
Female	1	4	0	5
Male	3	1	1	5
Total	4	5	1	10
Chi-Square of Pearson	p= 0.150			

Supplementary Table 28 *- Crosstab and Chi-square of Pearson for variable "Consumption of sour-taste related foods between genders"*

Gender	Sometimes	Frequently	Total
Female	1	4	5
Male	5	0	5
Total	6	4	10

Chi-Square of Pearson	p= 0.010

Supplementary Table 29 - Crosstab and Chi-square of Pearson for variable "Regular consumption of condiments between genders"

		Gender			
Condiments		Female	Male	Total	Chi-Square of Pearson
Salt	Yes	4	5	9	p=0.292
	No	1	0	1	
Sugar	Yes	1	3	4	p= 0.197
	No	4	2	6	
Paprika	Yes	2	1	3	p=0.490
	No	3	4	7	
Garlic	Yes	5	4	9	p=0.292
	No	0	1	1	
Lemon	Yes	3	2	5	p= 0.527
	No	2	3	5	
Curry	Yes	0	0	0	
	No	5	5	10	
Ginger	Yes	0	1	1	p= 0.292
	No	5	4	9	
Pepper	Yes	3	0	3	p= 0.038
	No	2	5	7	
Vinegar	Yes	5	1	6	p= 0.010
	No	0	4	4	
Chili pepper	Yes	1	1	2	p=1.000
	No	4	4	8	
Cumin	Yes	1	0	1	p=0.292
	No	4	5	9	
Cloves	Yes	0	0	0	
	No	5	5	10	
Nutmeg	Yes	1	0	1	p=0.292
	No	4	5	9	
Onion	Yes	5	4	9	p=0.292
	No	0	1	1	
Cinnamon	Yes	2	3	5	p= 0.490
	No	3	4	7	
Turmeric	Yes	1	0	1	p=0.292
	No	4	5	9	
Herbs	Yes	4	2	6	p=0.197
	No	1	3	4	
Soy Sauce	Yes	0	0	0	
	No	5	5	10	
Mustard	Yes	0	0	0	
	No	5	5	10	

Supplementary Table 30 - Crosstab and Chi-square of Pearson for variable "Frequency of consumption of liqueur wines in tasting"

Gender	Biweekly	Monthly	Total
Female	0	1	1
Male	1	3	4
Total	1	4	5
Chi-Square of Pearson	p= 0.576		

Supplementary Table 31 *– Crosstab and Chi-square of Pearson for variable "Frequency of consumption of calm wines in tasting"*

Gender	Daily	Total
Female	1	1
Male	4	4
Total	5	5
Chi-Square of Pearson	---	

Supplementary Table 32 *- Crosstab and Chi-square of Pearson for variable "Frequency of consumption of sparkling wines in tasting"*

Gender	Daily	Weekly	Total
Female	0	1	1
Male	3	1	4
Total	3	2	5
Chi-Square of Pearson	p= 0.171		

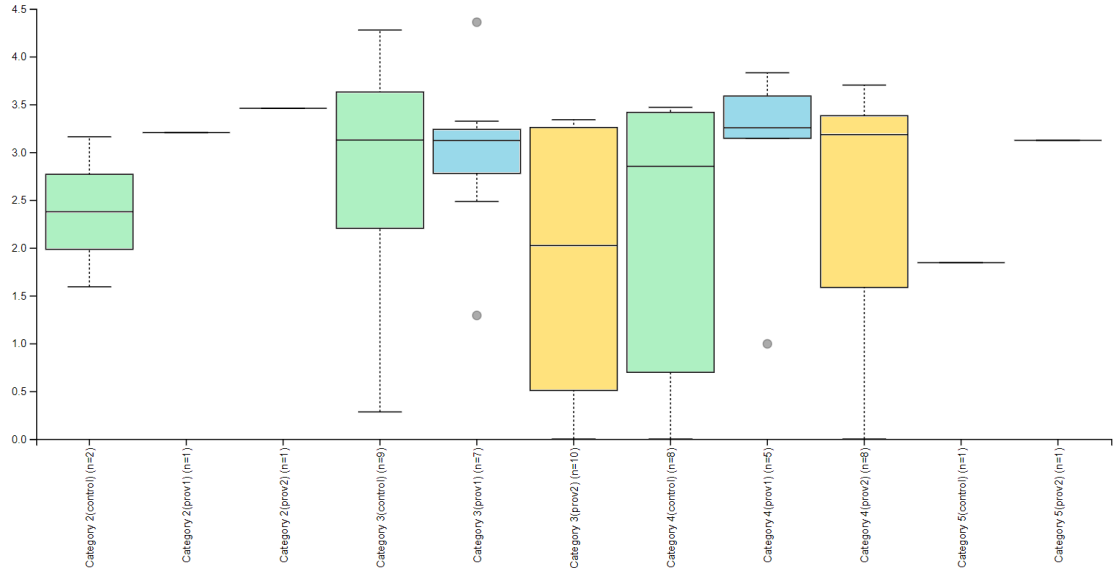
Supplementary Table 33 - *Crosstab and Chi-square of Pearson for variable "Frequency of wine tastings"*

Gender	5-6 timer per week	One time per day	Total
Female	0	1	1
Male	1	3	4
Total	1	4	5
Chi-Square of Pearson	p= 0.576		

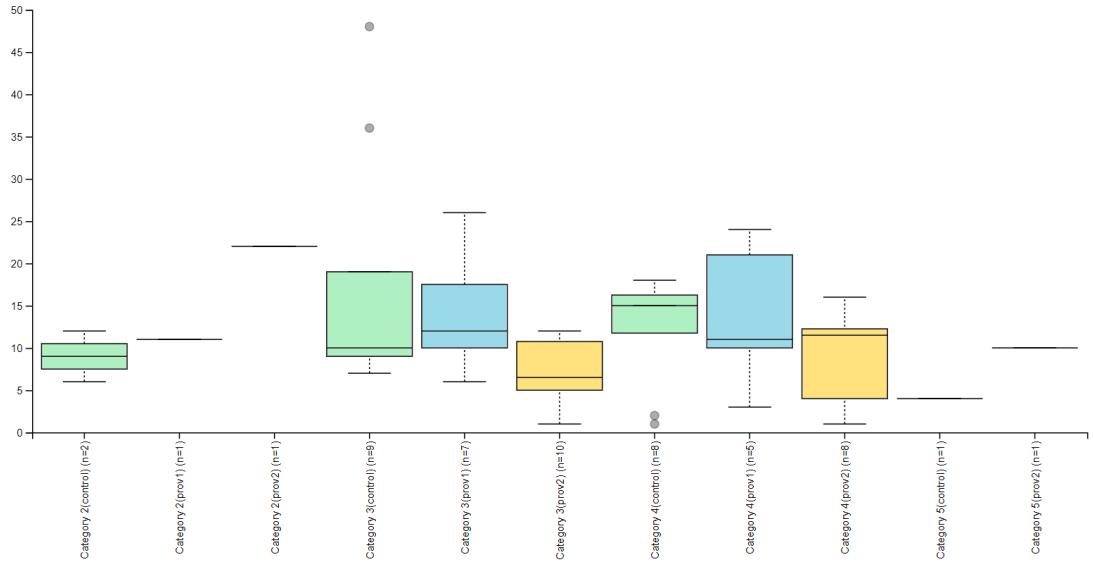
Supplementary Table 34 *- Crosstab and Chi-square of Pearson for variable "Higher sensibility to taste"*

Gender	Bitter	Equal to all tastes	Sweet+Sour	Total
Female	0	1	0	1
Male	2	0	2	4
Total	2	1	2	5
Chi-Square of Pearson	p= 0.082			

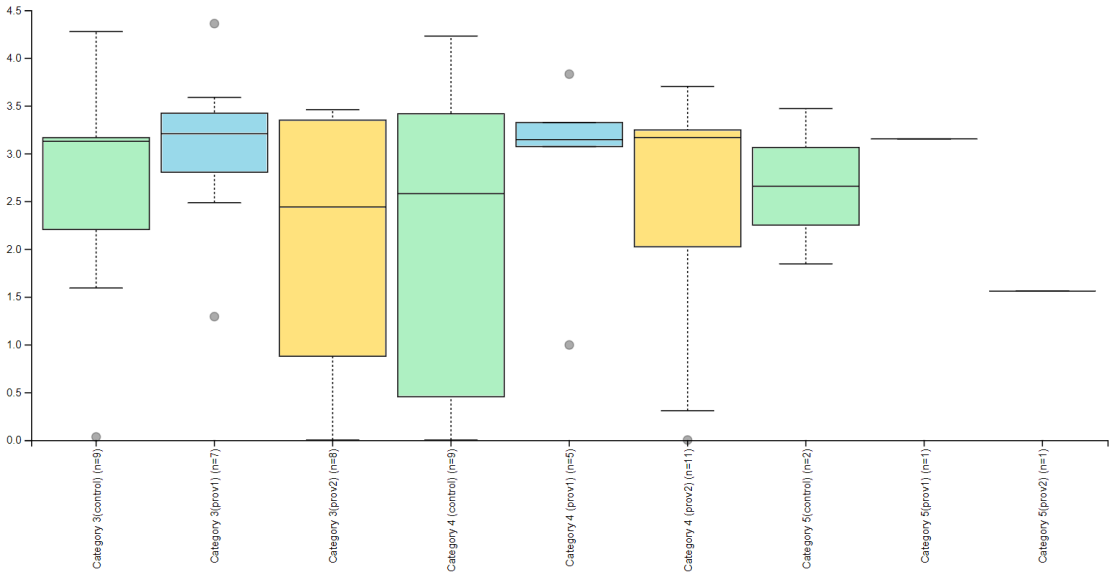
Oral Microbiome



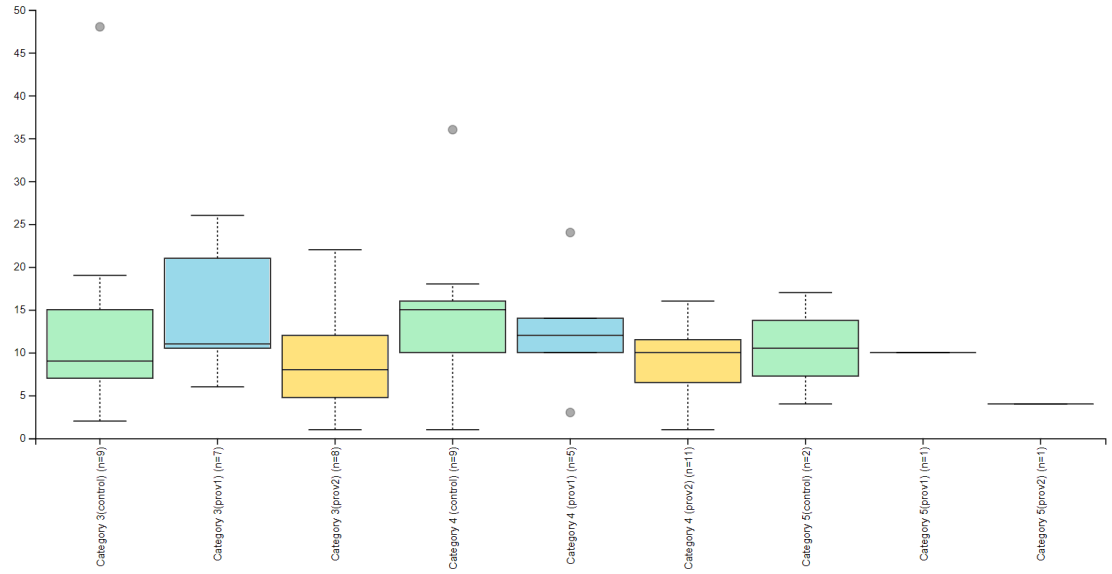
Supplementary Figure 1 - Boxplot charts depicting the distribution of the Shannon index in the different categories of sour taste for each group (mean: control (cat2 – 2.4, cat3 – 2.6, cat4 – 2.4, cat5 – 1.8); prov1(cat2 – 3.2, cat3 – 2.9, cat4 – 2.9); prov2(cat2 – 3.4, cat3 – 2.0, cat4 – 2.8, cat5 – 3.1; p=0.86), control samples in green, prov1 in blue and prov2 in yellow.



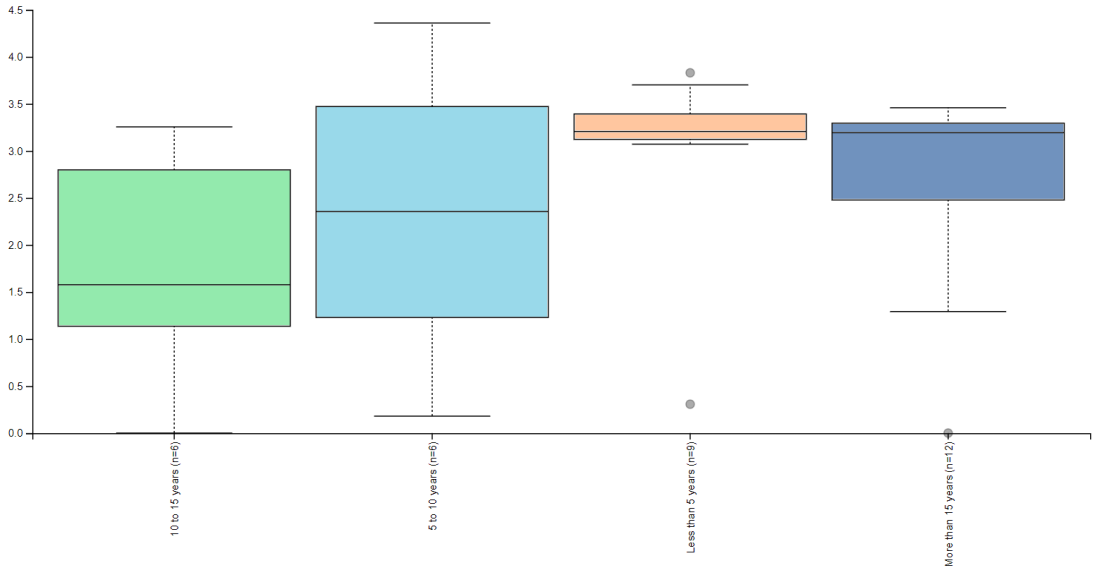
Supplementary Figure 2 - Boxplot charts depicting the distribution of the ASVs counts in the different categories of sour taste for each group (mean: control (cat2 – 9, cat3 – 17.7, cat4 – 12.4, cat5 – 4); prov1(cat3 – 14.1, cat4 – 13.8); prov2(cat2 – 22, cat3 – 7.3, cat4 – 9.1; p=0.36), control samples in green, prov1 in blue and prov2 in yellow.



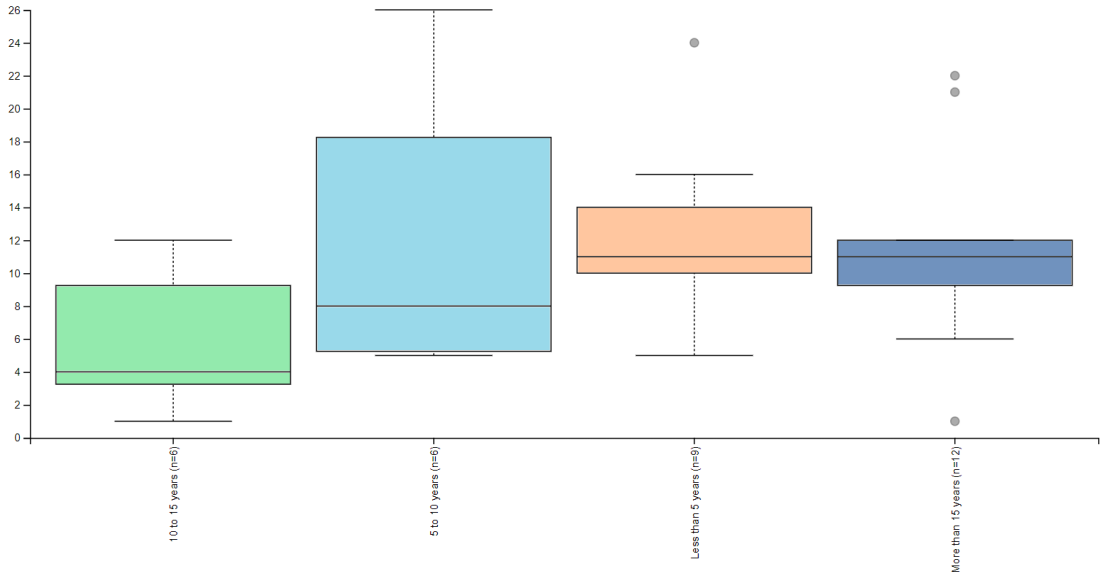
Supplementary Figure 4 - Boxplot charts depicting the distribution of the Shannon index in the different categories of astringent taste for each group (mean: control (cat3 – 2.63, cat4 – 2.3, cat5 – 2.6); prov1(cat3 – 3.0, cat4 – 2.9, cat5 – 3.1); prov2(cat3 – 2.1, cat4 – 2.7, cat5 – 1.5); p=0.94), control samples in green, prov1 in blue and prov2 in yellow.



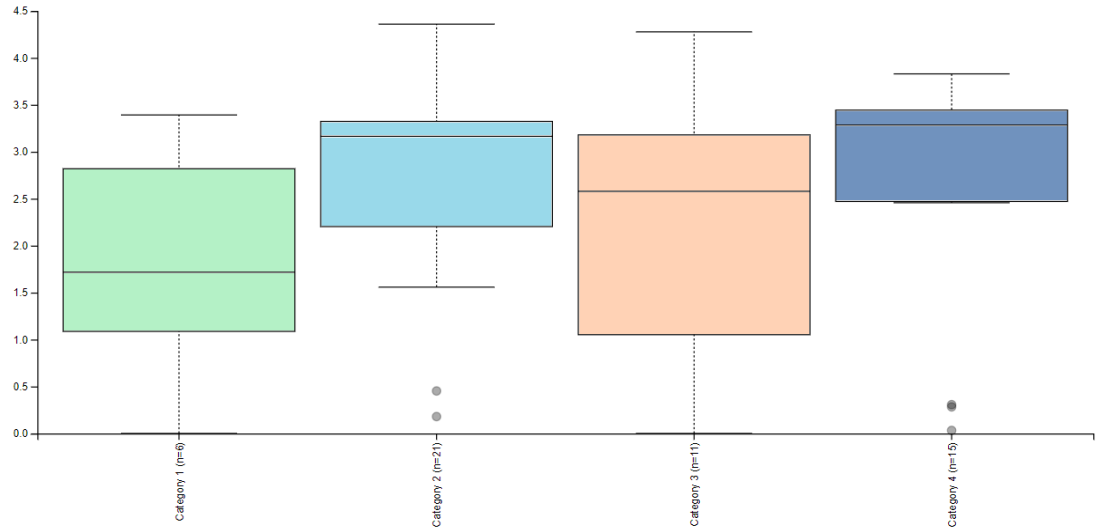
Supplementary Figure 3 - Boxplot charts depicting the distribution of the ASVs counts in the different categories of astringent taste for each group (mean: control (cat3 – 13.8, cat4 – 15, cat5 – 10.5); prov1(cat3 – 15.1, cat4 – 10.5, cat5 – 10); prov2(cat3 – 7.1, cat4 – 9.3, cat5 – 4); p=0.57), control samples in green, prov1 in blue and prov2 in yellow.



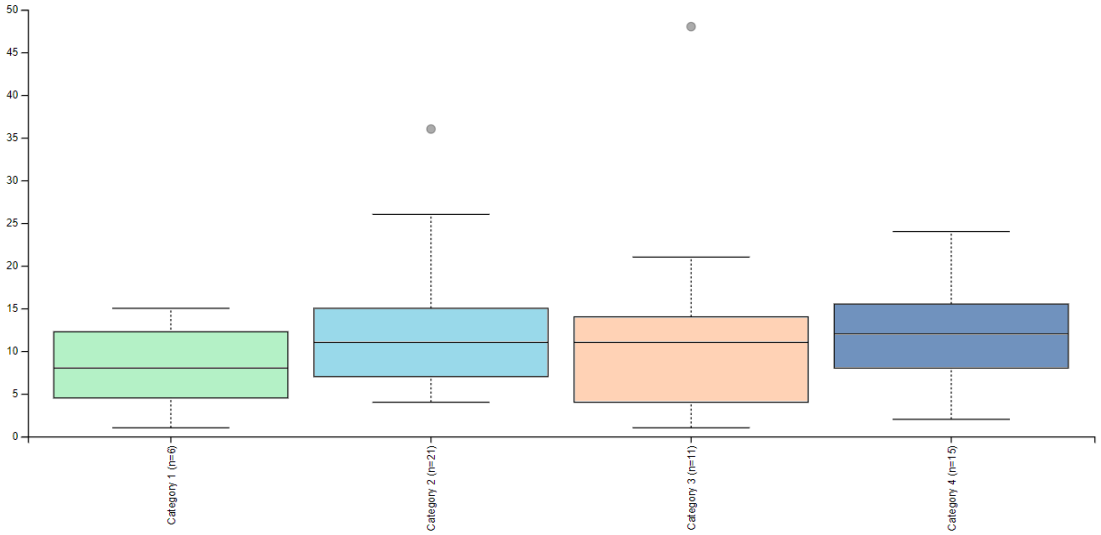
Supplementary Figure 5 - Boxplot charts depicting the distribution of the Shannon index in the different categories of career duration of wine taster for all subjects (mean: less than 5 years – 3.01, 5 to 10 years – 2.3, 10 to 15 years – 2.1, more than 15 years – 2.9; p=0.29), 10 to 15 years in green, 5 to 10 years in blue, less than 5 years in orange and more than 15 years in dark blue.



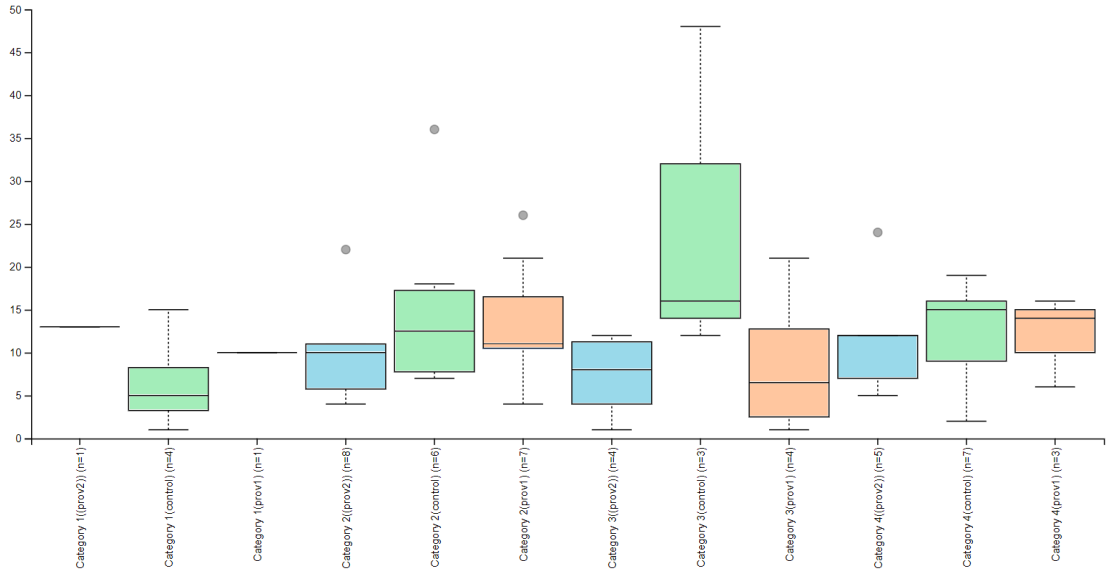
Supplementary Figure 6 - Boxplot charts depicting the distribution of the ASV abundance in the different categories of career duration of wine taster for all subjects (mean: less than 5 years – 12.7, 5 to 10 years – 12.1, 10 to 15 years – 5.8, more than 15 years – 11.2, p=0.14), 10 to 15 years in green, 5 to 10 years in blue, less than 5 years in orange and more than 15 years in dark blue.



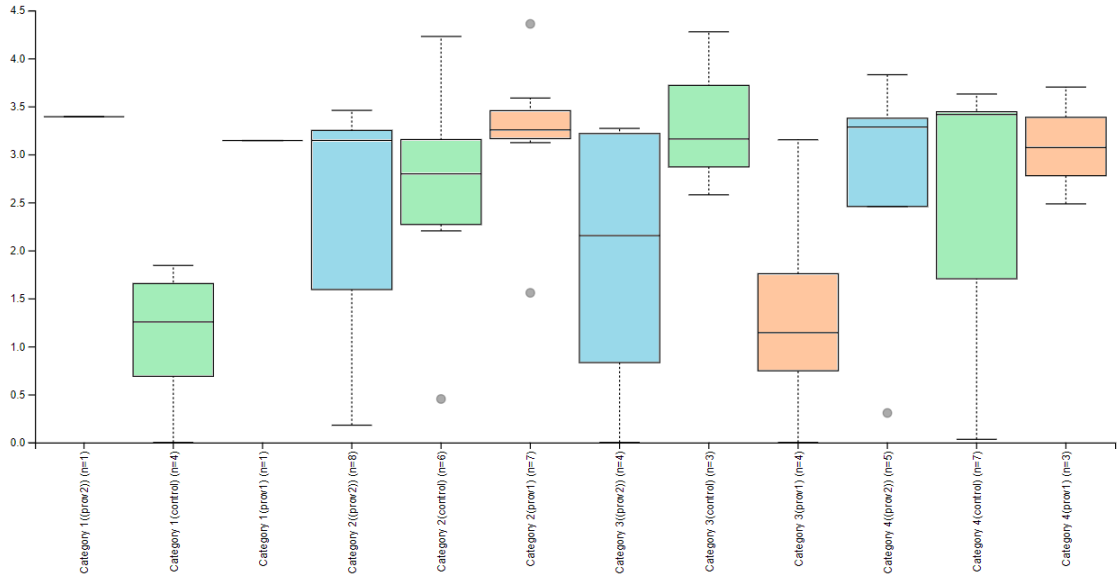
Supplementary Figure 7 - Boxplot charts depicting the distribution of the Shannon index in the different categories alcohol consumption (mean: cat1 – 2.2, cat2 – 2.7, cat3 – 2.5, cat4 – 2.6; p=0.22), category 1 in green, category 2 in light blue, category 3 in orange and category 4 in dark blue.



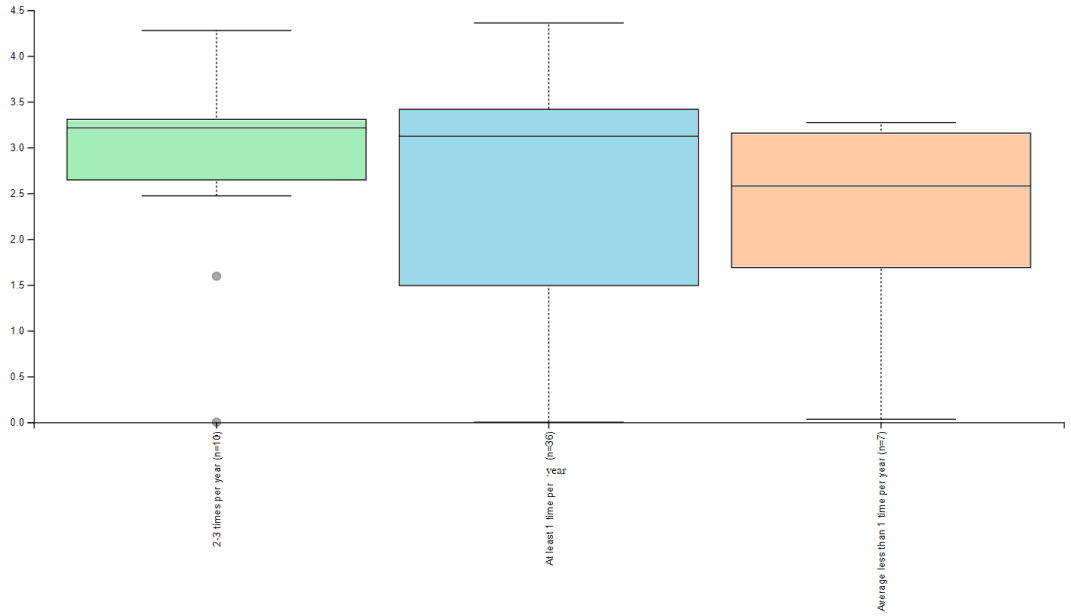
Supplementary Figure 8 - Boxplot charts depicting the distribution of the ASV abundance in the different categories alcohol consumption (mean: cat1 – 8.1, cat2 – 12.7, cat3 – 12.7, cat4 – 12.1; p=0.59), category 1 in green, category 2 in light blue, category 3 in orange and category 4 in dark blue.



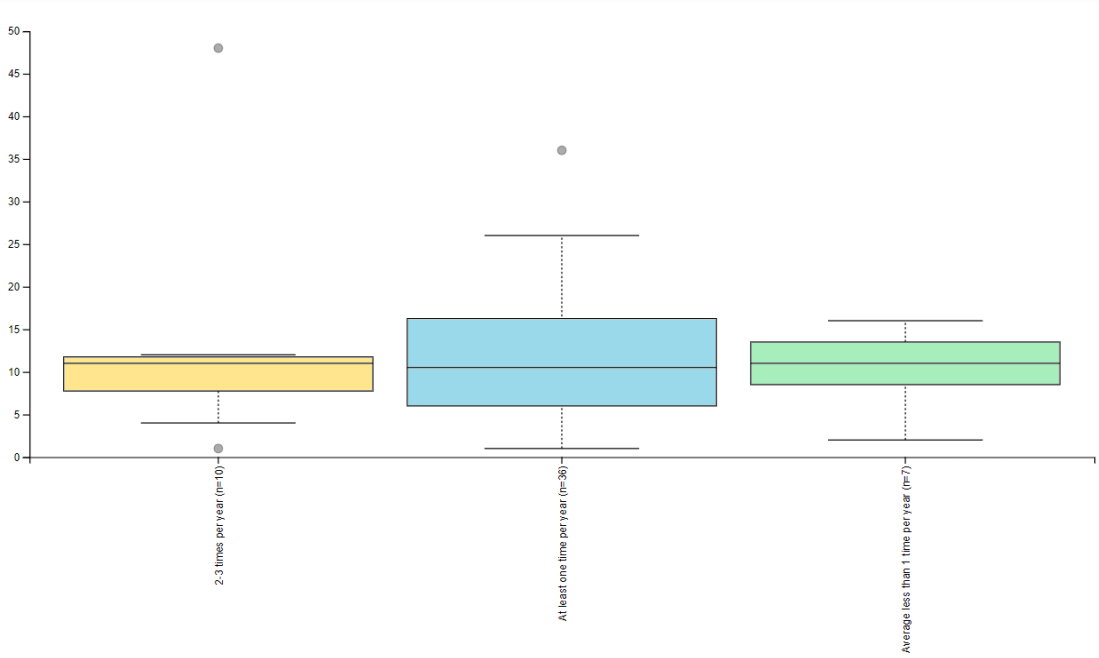
Supplementary Figure 9 - Boxplot charts depicting the distribution of the Shannon index in the different categories of alcohol consumption for each group (mean: control (cat1 – 1.45, cat2 – 2.6, cat.3 – 3.3, cat.4 – 2.5), prov1 (cat.1 – 3.1, cat.2 – 3.5, cat.3 – 1.8, cat.4 – 3.1), prov2 (cat.1 – 3.4, cat.2 – 2.4, cat.3 – 2.5, cat.4 – 2.6; p= 0.29), control samples in green, prov1 in orange and prov2 in blue.



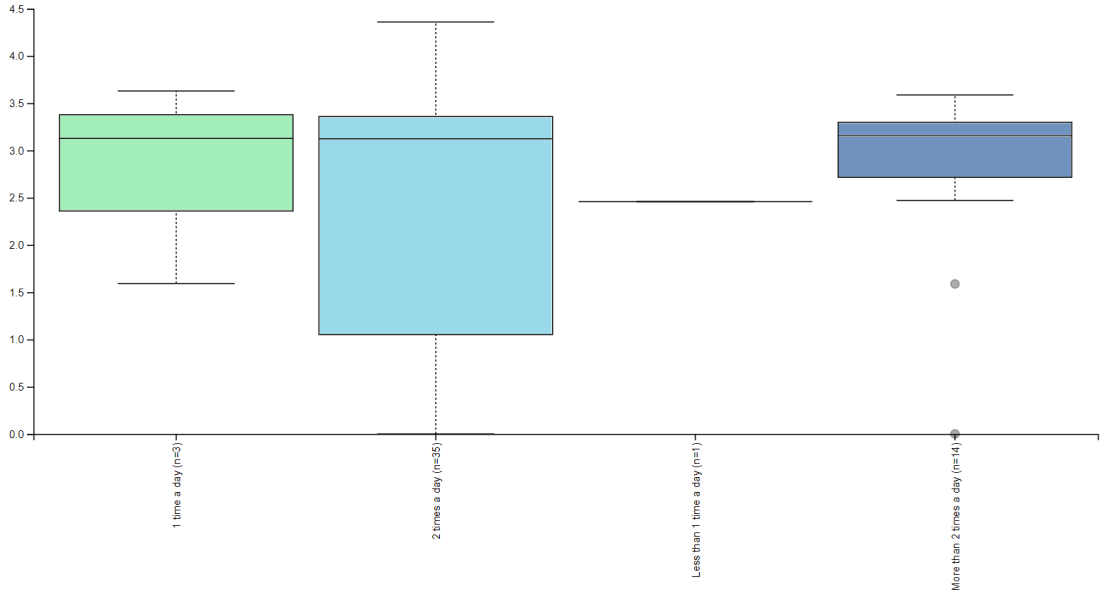
Supplementary Figure 10 - Boxplot charts depicting the distribution of the ASV abundance in the different categories of alcohol consumption for each group (mean: control (cat1 – 6.5, cat2 – 15.5, cat.3 – 25.3, cat.4 – 12.3), prov1 (cat.1 – 10, cat.2 – 14.1, cat.3 – 8.8, cat.4 – 12), prov2 (cat.1 – 13, cat.2 – 9.7, cat.3 – 7.3, cat.4 – 12; p= 0.70), control samples in green, prov1 in orange and prov2 in blue.



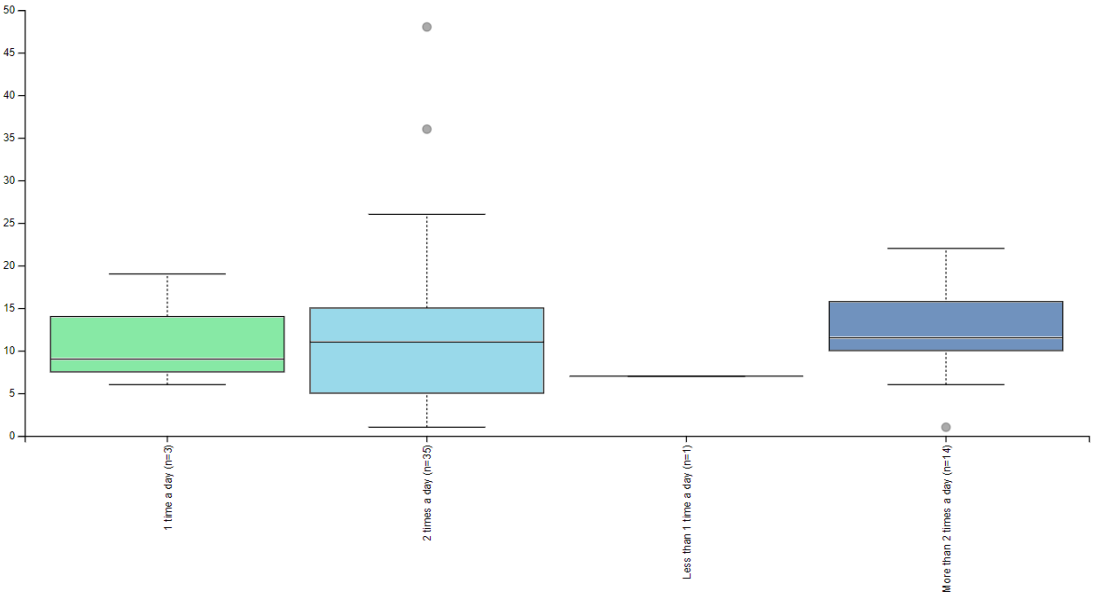
Supplementary Figure 11 - Boxplot charts depicting the distribution of the Shannon index in the different frequencies of attendance to the dentist (mean: At least one time per year – 2.6, 2-3 times per year – 3.1, Average less than one time per year – 2.2; p= 0.47); 2-3 times per year in green, at least 1 time per year in blue and average than 1 time per year in orange.



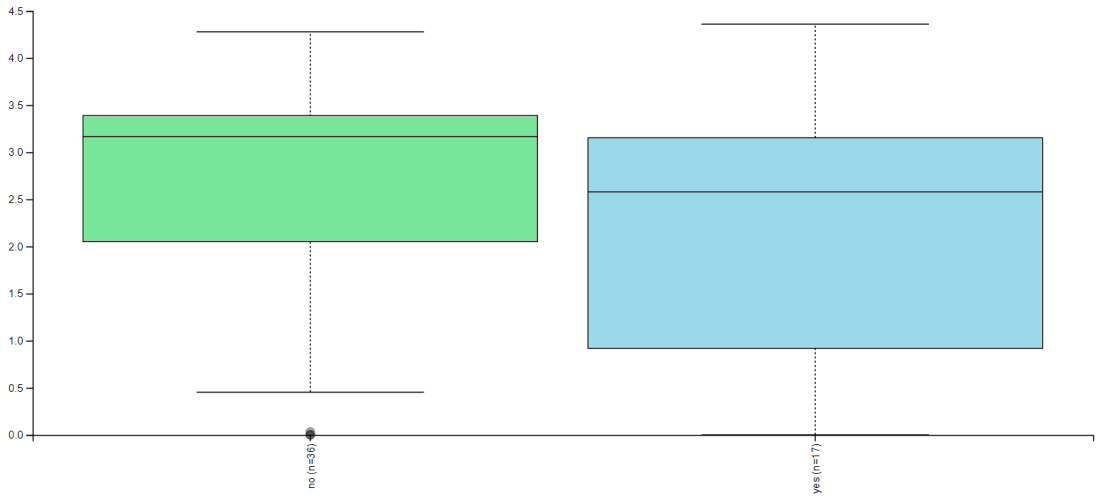
Supplementary Figure 12 - Boxplot charts depicting the distribution of the ASV abundance in the different frequencies of attendance to the dentist (mean: At least one time per year – 12.2, 2-3 times per year – 12.7, Average less than one time per year – 9.6; p= 0.89), 2-3 times per year in yellow at least 1 time per year in blue and average than 1 time per year in green.



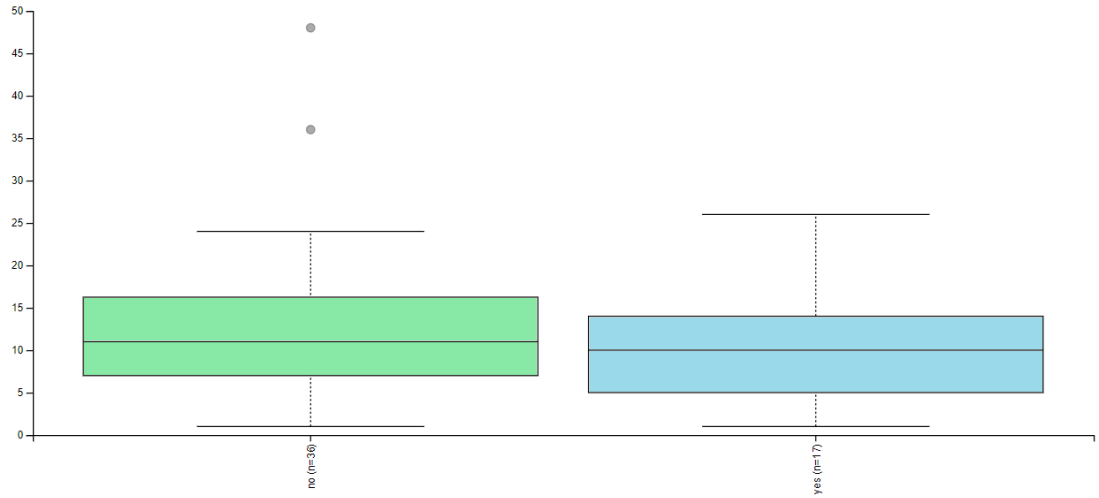
Supplementary Figure 13 - Boxplot charts depicting the distribution of the Shannon index in the different frequencies of teeth brushing (Less than one time a day – 2.45, 1 time a day – 2.8, 2 times a day – 2.5, more than 2 times a day – 3.0; p= 0.89); 1 time a day in green, 2 times a day in light blue, more than 2 times a day in dark blue.



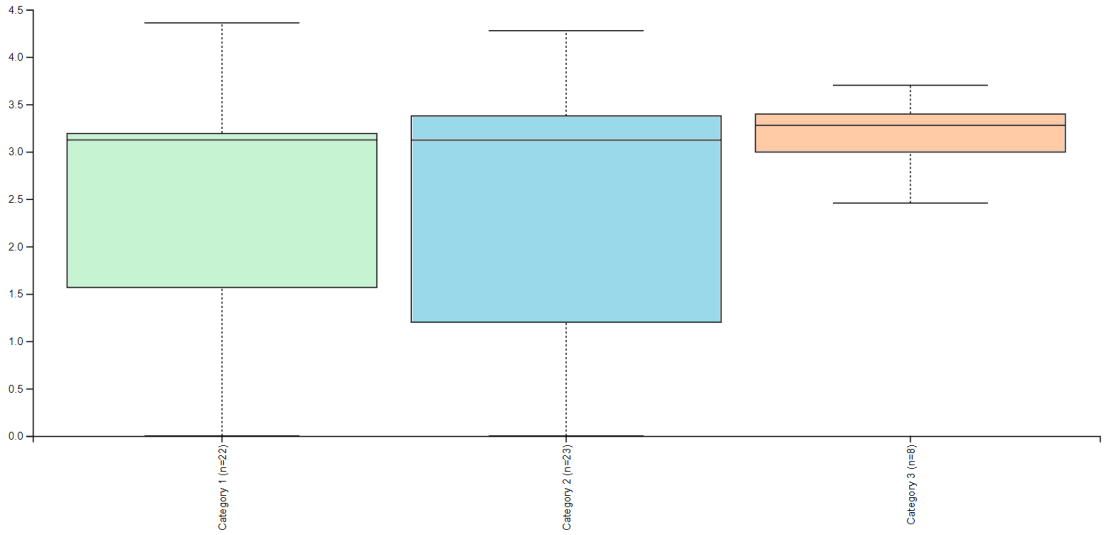
Supplementary Figure 14 - Boxplot charts depicting the distribution of the ASV abundance in the different frequencies of teeth brushing (Less than one time a day – 7, 1 time a day – 11.3, 2 times a day – 12.1, more than 2 times a day – 12.3; p= 0.77); 1 time a day in green, 2 times a day in light blue, more than 2 times a day in dark blue.



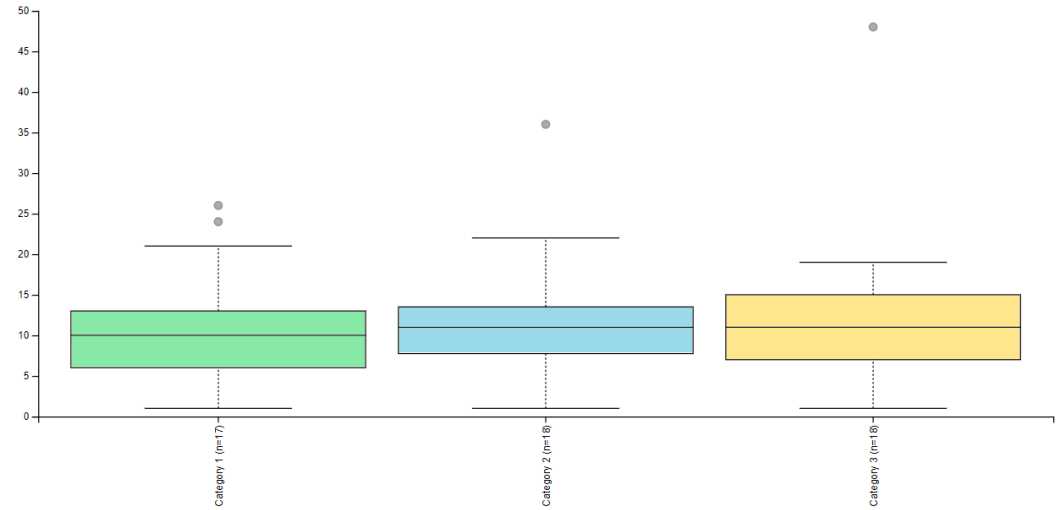
Supplementary Figure 15 - Boxplot charts depicting the distribution of the Shannon index in the use of mouthwash (mean: users – 2.4, non-users – 2.8; p= 0.09); non-users in green and users in blue.



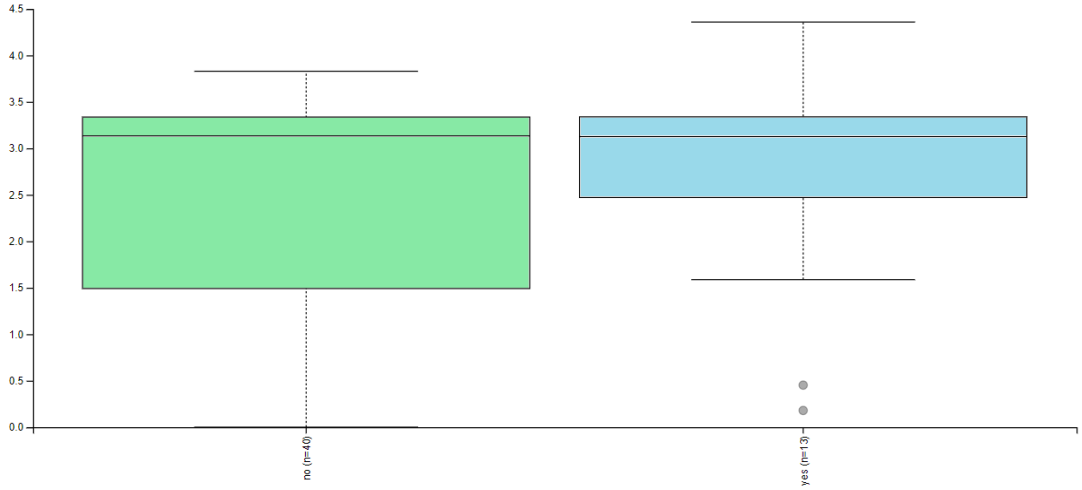
Supplementary Figure 16 - Boxplot charts depicting the distribution of the ASV abundance in the use of mouthwash (mean: users – 10.5, non-users – 12.9, p= 0.29); non-users in green and users in blue.



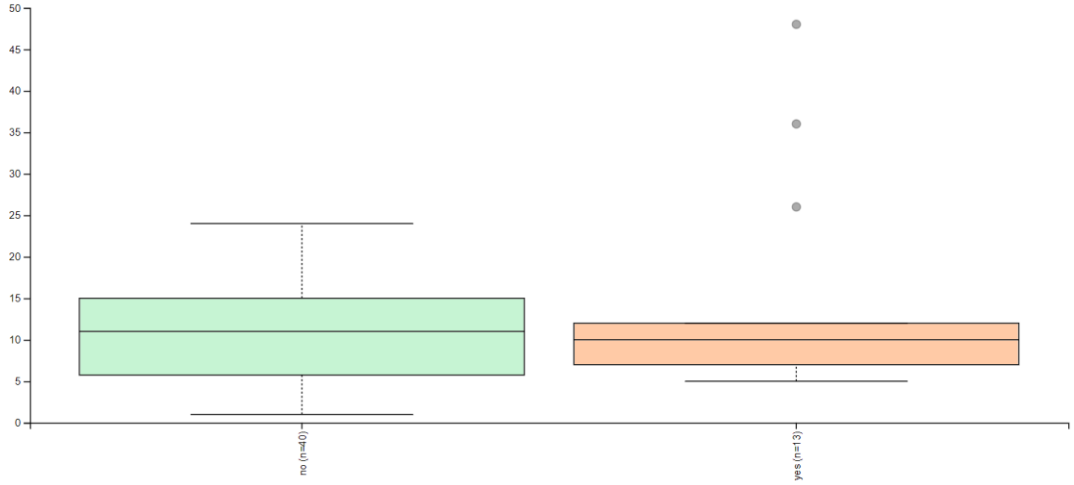
Supplementary Figure 17 - Boxplot charts depicting the distribution of the Shannon index in the different categories of age (mean: cat.1 – 2.8, cat2 – 2.7, cat.3 – 2.3; p= 0.41); category 1 in green, category 2 in blue and category 3 in orange.



Supplementary Figure 18 - Boxplot charts depicting the distribution of the ASV abundance in the different categories of age (mean: cat.1 – 11.2, cat2 – 12.1, cat.3 – 12.6; p= 0.93), category 1 in green, category 2 in blue and category 3 in yellow.

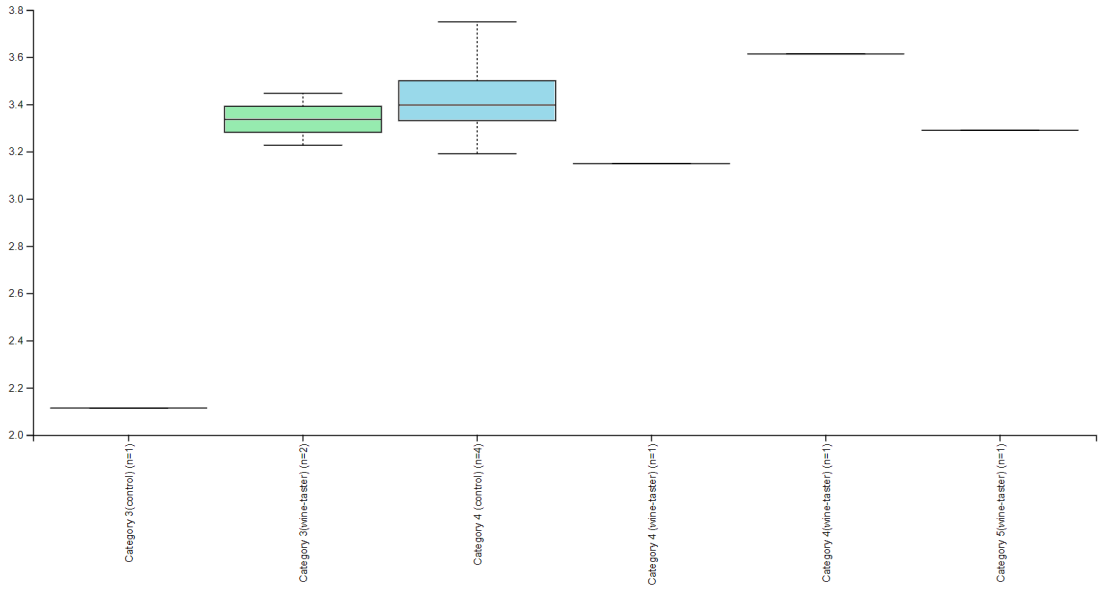


Supplementary Figure 19 - Boxplot charts depicting the distribution of the Shannon index in smokers and non-smokers (mean: non-smokers – 2.6, smokers – 2.7; p= 0.45); non-smokers in green and smokers in blue.

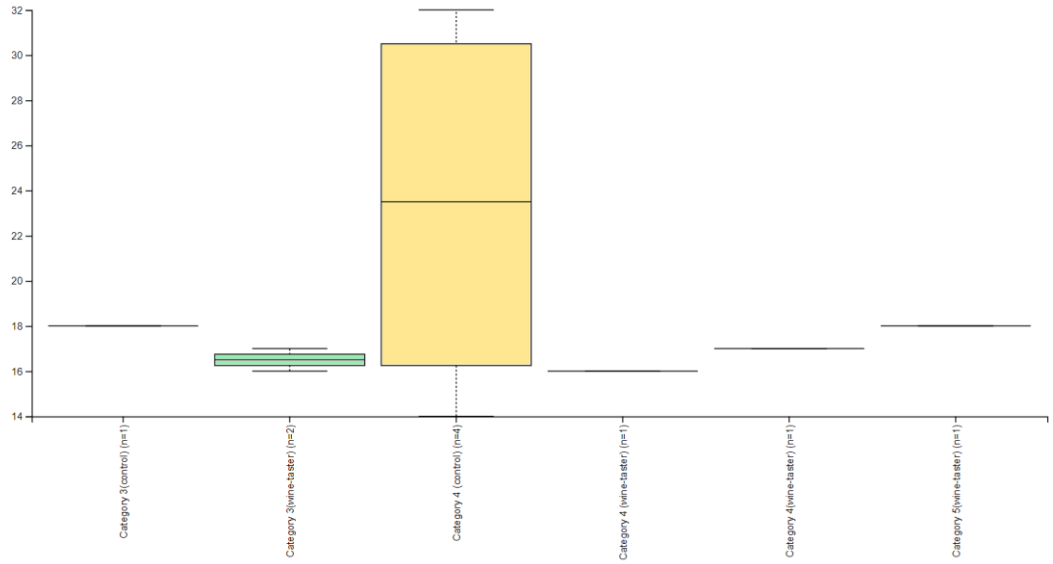


Supplementary Figure 20 - Boxplot charts depicting the distribution of the ASV abundance in smokers and non-smokers (mean: non-smokers – 11, smokers – 15.2; p= 0.84); non-smokers in green and smokers in orange.

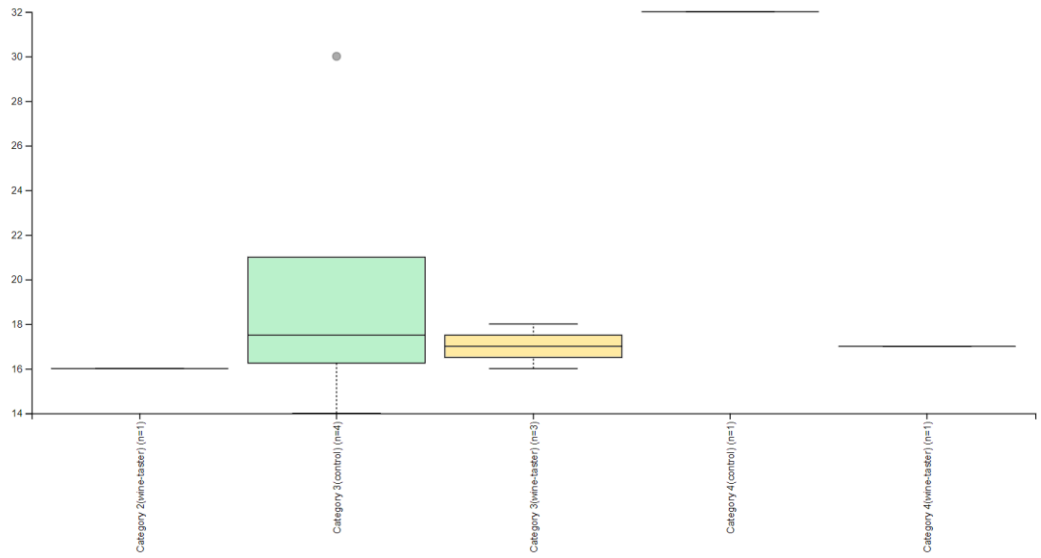
Nasal Microbiome



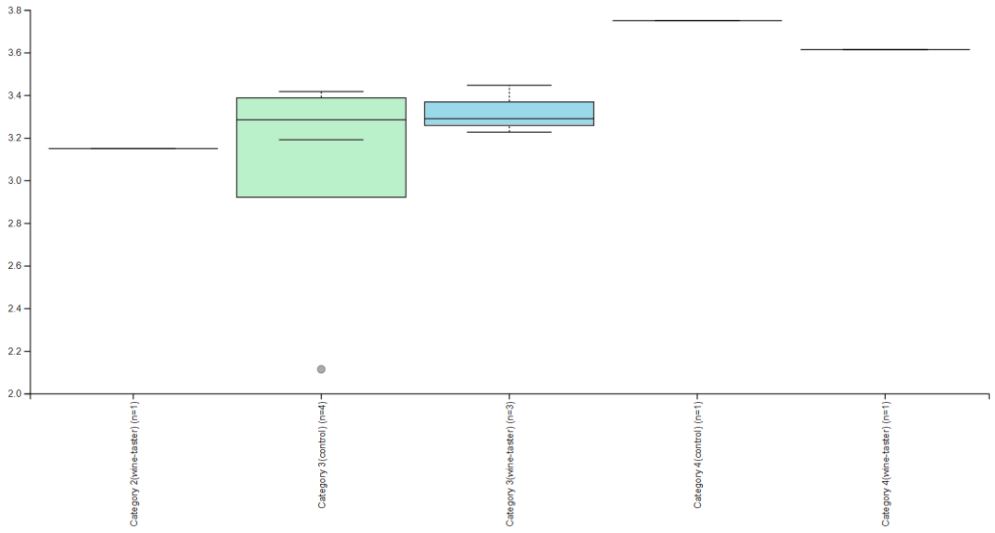
Supplementary Figure 21 - Boxplot charts depicting the distribution of the Shannon index in the different categories of astringent taste for each group (mean: control (cat3 – 2.1, cat4 – 3.4); wine taster (cat3 – 3.3, cat4 – 3.4, cat5 – 3.3); p=0.37, control samples in blue and wine tasters in green.



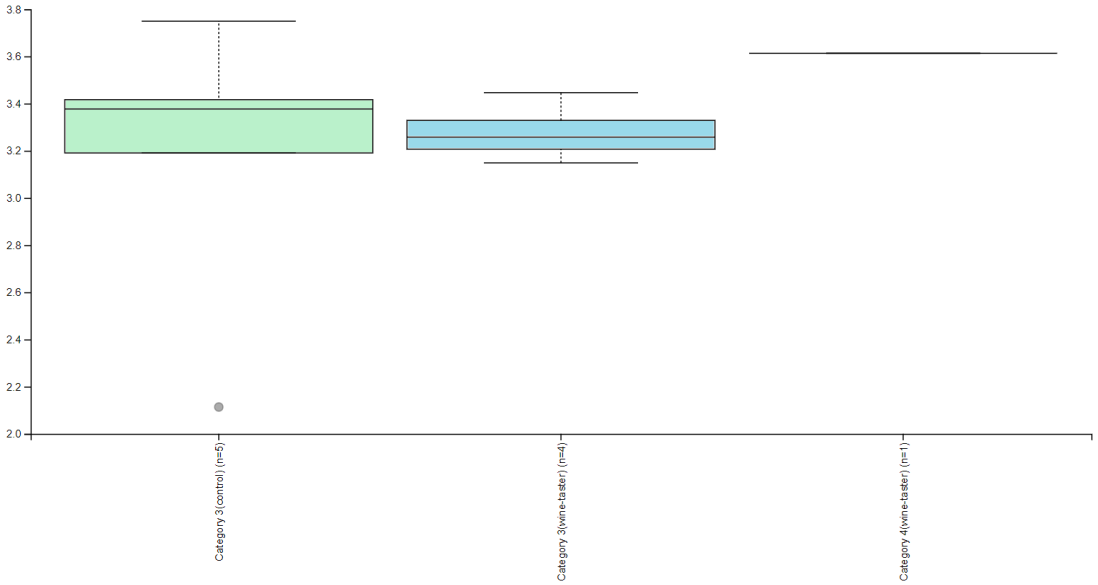
Supplementary Figure 22 - Boxplot charts depicting the distribution of ASV abundance in the different categories of astringent taste for each group (mean: control (cat3 – 18, cat4 – 23.3); wine taster (cat3 – 16.5, cat4 – 16.5, cat5 - 18); p=0.72, control samples in orange and wine tasters' in green.



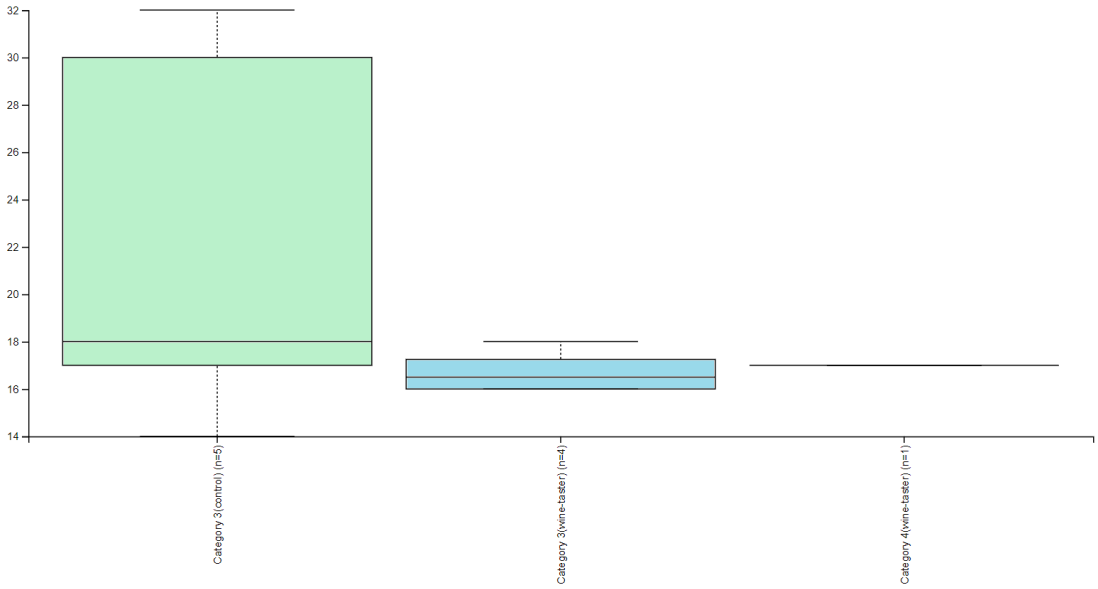
Supplementary Figure 23 - Boxplot charts depicting the distribution of the Shannon index in the different categories of bitter taste for each group (mean: control (cat3 – 3.0, cat4 – 3.7); wine taster (cat2 – 3.1, cat3 – 3.3, cat4 – 3.6); p=0.23, control samples in green and wine tasters in blue.



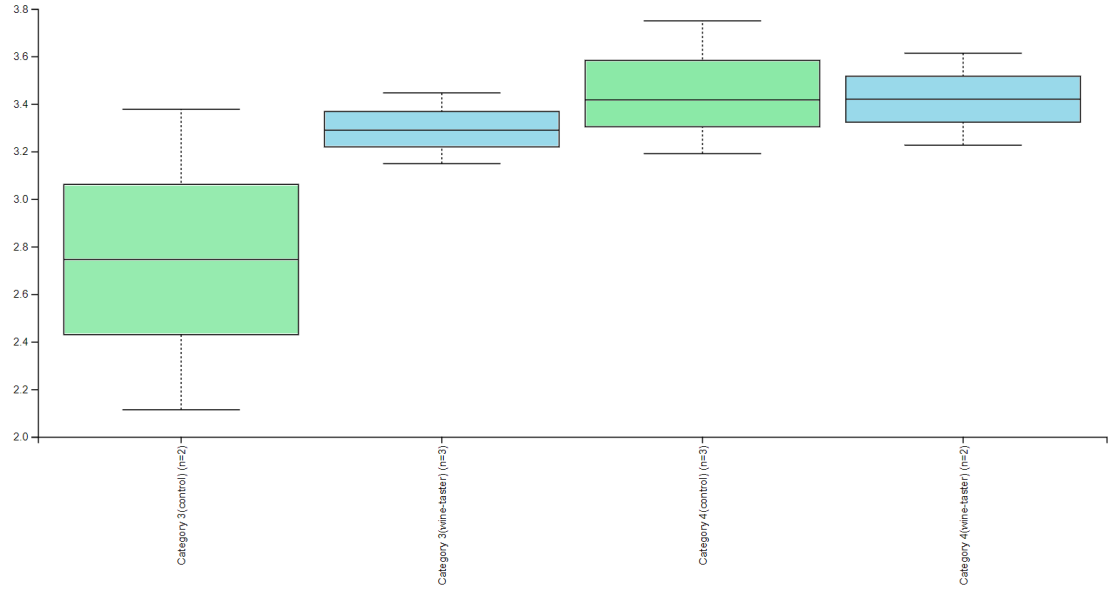
Supplementary Figure 24 - Boxplot charts depicting the distribution of ASV abundance in the different categories of bitter taste for each group (mean: control (cat3 – 19.8, cat4 – 32); wine taster (cat2 – 16, cat3 – 17, cat4 – 17); p=0.49, control samples in green and wine tasters in orange.



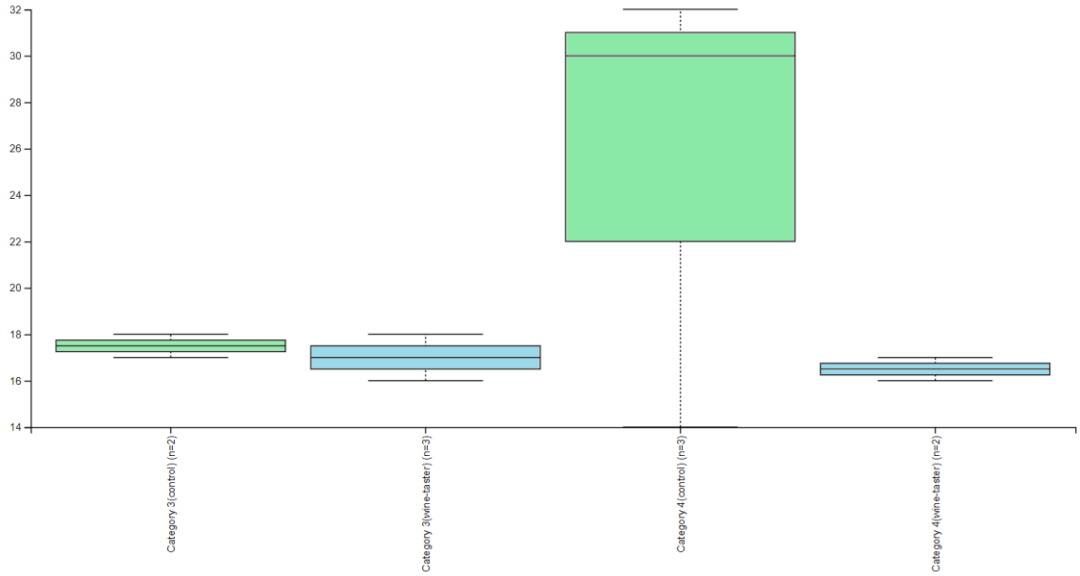
Supplementary Figure 25 - Boxplot charts depicting the distribution of Shannon index in the different categories of sweet taste for each group; (mean: control (cat3 – 3.3, cat4 – 3.6), wine tasters (cat3 – 3.2); p=0.45), control samples in green and wine tasters in blue.



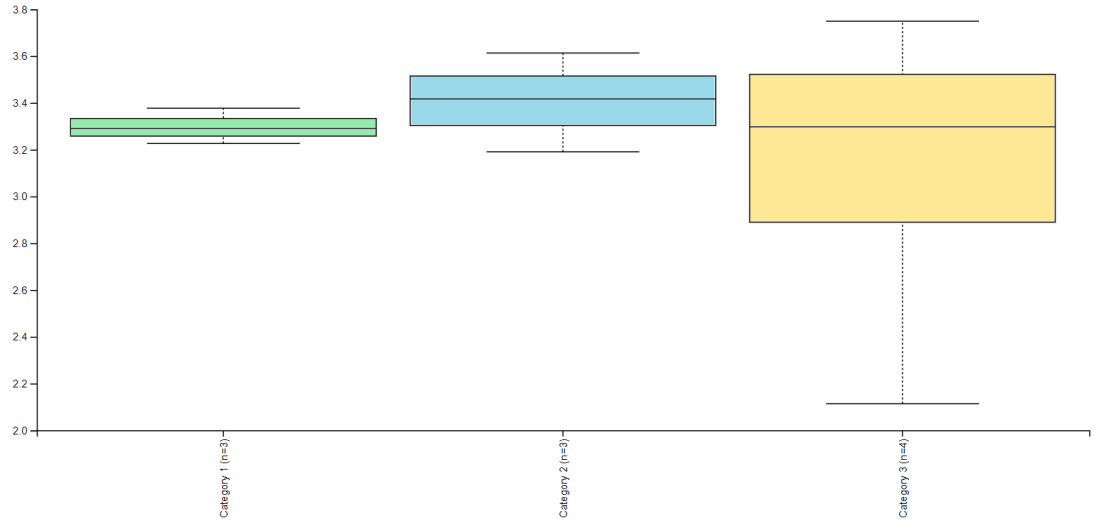
Supplementary Figure 26 - Boxplot charts depicting the distribution of ASV abundance in the different categories of sweet taste for each group; (mean: control (cat3 – 16.8, cat4 - 17), wine tasters (cat3 – 22.2); p=0.55), control samples in green and wine tasters in blue.



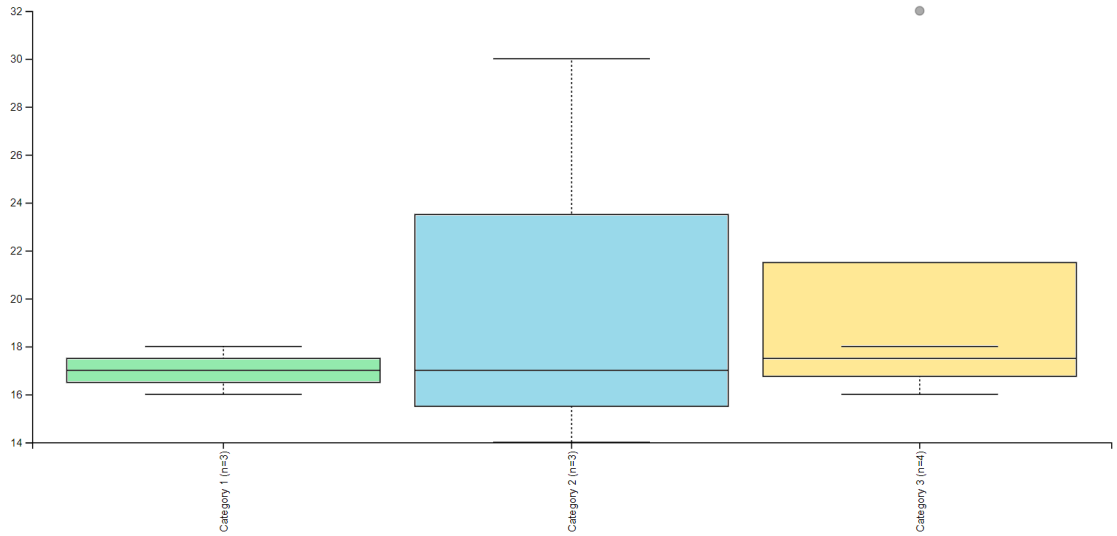
Supplementary Figure 27 - Boxplot charts depicting the distribution of Shannon index in the different categories of sour taste for each group (mean: control (cat3 – 2.7, cat4 – 3.4), wine tasters (cat3 – 3.3, cat4 – 3.4), p=0.65), control samples in green, wine tasters in blue.



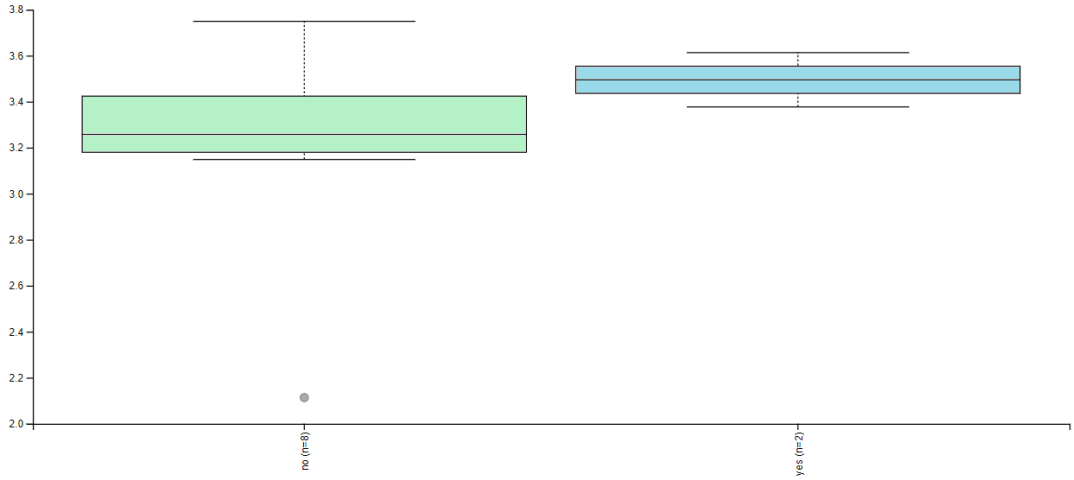
Supplementary Figure 28 - Boxplot charts depicting the distribution of ASV abundance in the different categories of sour taste for each group; (mean: control (cat3 – 17.5, cat4 – 25.3), wine tasters (cat3 – 17, cat4 – 16.5); p=0.71), control samples in green, wine tasters in blue.



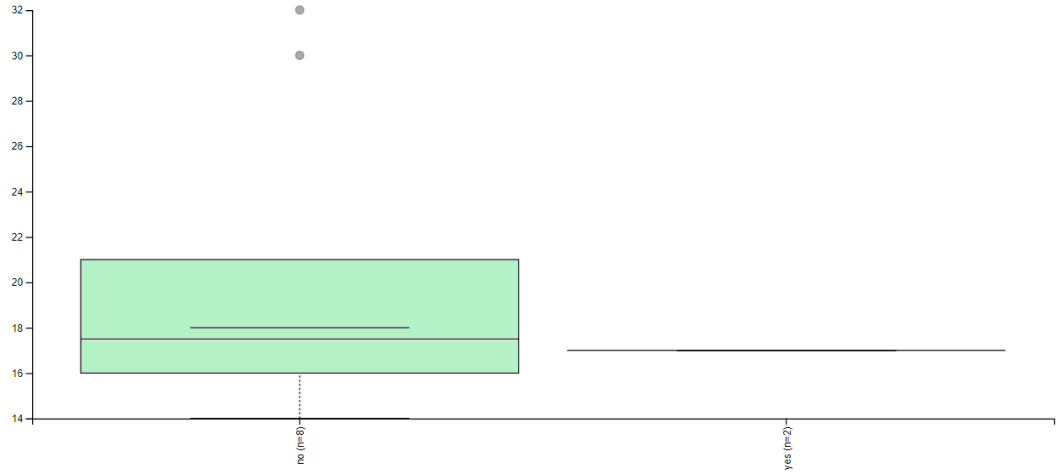
Supplementary Figure 29 - Boxplot charts depicting the distribution of Shannon index in the different categories of age (mean: cat1 – 3.3, cat2 – 3.4, cat3 – 3.1; p=0.84), category 1 in green, category 2 in blue and category 3 in yellow.



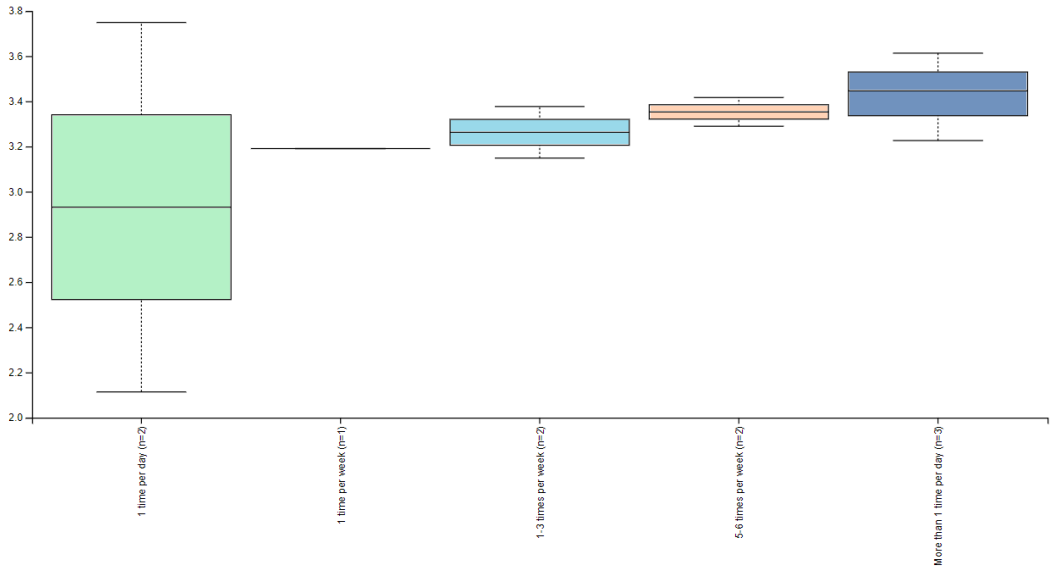
Supplementary Figure 30 - Boxplot charts depicting the distribution of ASV abundance in the different categories of age; (mean: cat1 – 17, cat2 – 20.3, cat3 – 20.8; p=0.80), category 1 in green, category 2 in blue and category 3 in yellow.



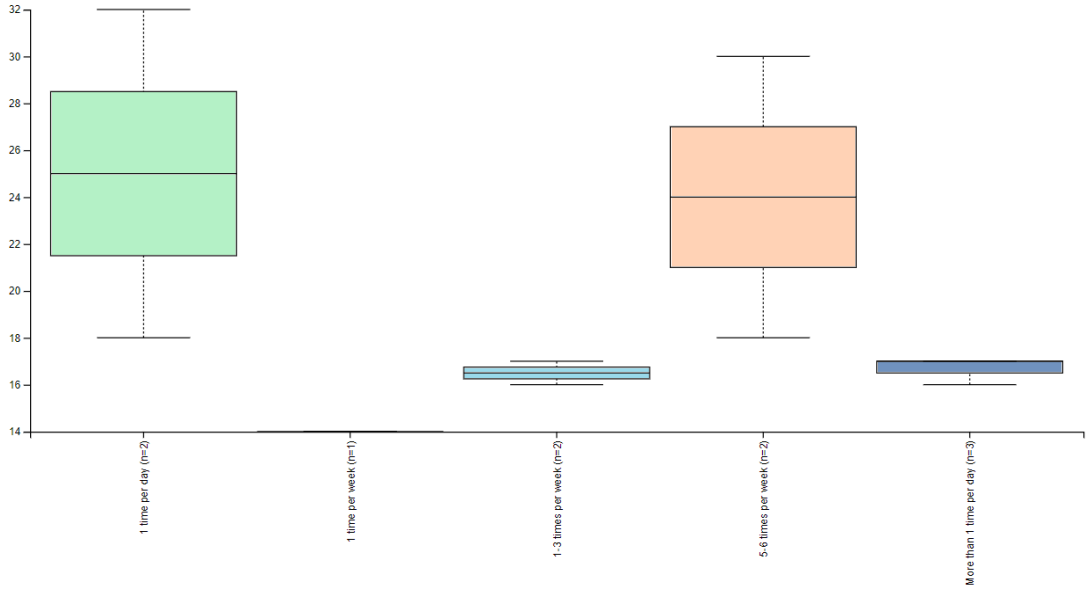
Supplementary Figure 31 - Boxplot charts depicting the distribution of Shannon index in the different smokers and non-smokers; (mean: non-smokers – 3.2, smokers – 3.5; p=0.29), non-smokers in green, smokers in blue.



Supplementary Figure 32 - Boxplot charts depicting the distribution of ASV abundance in the different smokers and non-smokers; (mean: non-smokers – 20.1, smokers – 17; p=0.79), non-smokers in green, smokers in blue.



Supplementary Figure 33 - Boxplot charts depicting the distribution of Shannon index in the different frequencies of alcohol consumption between all subjects; (mean: one time per day – 2.9, one time per week – 3.2, 1-3 time per week – 3.3, 5-6 time per week – 3.4, more than one time per day – 3.4; p=0.74), one time per day in green, 1-3 time per week in blue, 5-6 time per week in orange and more than one time per day in dark blue.



Supplementary Figure 34 - Boxplot charts depicting the distribution of ASV abundance in the different frequencies of alcohol consumption between all subjects; (mean: one time per day – 25, one time per week – 14, 1-3 time per week – 16.5, 5-6 time per week – 24, more than one time per day – 16.7; p=0.10), one time per day in green, 1-3 time per week in blue, 5-6 time per week in orange and more than one time per day in dark blue.