CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

PROFILING OF THE ORAL CAVITY MICROBIOTA OF YOUNG ADULTS AND THE POSSIBLE INFLUENCE OF THEIR HABITS

by

Maria Inês Leão Silva

February 2023

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Dissertation presented to *Escola Superior de Biotecnologia* of the
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Resumo

O interesse sobre o microbioma humano tem aumentado nos últimos anos. O microbioma oral inclui uma ampla diversidade microbiana, relacionada com o ambiente, fisiologia e hábitos de cada indivíduo, e com impacto no estado de saúde. Neste estudo, avaliou-se a prevalência de diferentes grupos bacterianos em jovens adultos que reportavam diferentes hábitos quotidianos. O objetivo foi o de compreender se a prevalência de grupos bacterianos maioritários são semelhantes, ao nível de filo ou de classe, entre indivíduos ou, se quando diferiam, tal poderia ser associado com comportamentos específicos. Uma vez que o estudo visava quantificar grupos taxonómicos específicos, optou-se pela técnica quantitativa de reação de polimerização em cadeia (*Polymerase Chain Reaction*, qPCR). O estudo incluiu amostras de saliva doadas por um grupo anónimo de 51 jovens adultos, estudantes de medicina dentária, que, simultaneamente, preencheram um inquérito sobre comportamentos e hábitos diários. Os participantes foram informados da natureza e objetivos do estudo ao qual deram o seu consentimento. Com base na extração de DNA total, foi quantificada a abundância de bactérias totais e dos filos *Bacteroidota*, *Bacillota* e *Actinomycetota* e das classes *Gamma-* e *Betaproteobacteria.* Em todos os casos, o gene 16S rRNA foi o alvo, utilizando-se sequências específicas para diferentes grupos bacterianos. A abundância do gene rRNA 16S por volume de saliva variou entre 7.3 e 10.0 log nº cópias/mL. O filo *Bacteroidota* foi observado como sendo o mais abundante com 6.3 a 9.3 log nº cópias/mL, sendo o filo *Bacillota* o que apresentava abundância mais baixa, com 5.5 e 8.1 log nº cópias/mL. De um conjunto de 19 variáveis relacionadas com características pessoais e hábitos diários que foram estruturados numa escala binária (presente/ausente ou frequente/raro), observou-se que o sexo, o consumo de antibiótico nos 12 meses que antecederam o estudo, o uso de pastilha elástica, os hábitos de onicofagia ou de morder os lápis ou canetas estavam associados com diferenças significativas na abundância de alguns grupos bacterianos. Estas diferenças foram mais notórias nos casos de Bacillota, *Bacteroidota, Actinomycetota* e *Gammaproteobacteria* e sexo e toma de antibiótico, *Betaproteobacteria* e CPO e uso de pastilha elástica e outros grupos bacterianos e onicofagia e roer de canetas. Embora não se possa estabelecer uma relação de causa-efeito nestas associações, é possível inferir que alguns hábitos específicos podem contribuir para modelar a microbiota da cavidade oral.

Palavras-chave: microbiota oral; qPCR; saliva; *Actinomycetota; Bacteroidota; Bacillota; Pseudomonadota*

Abstract

The interest in the human microbiome is increasing in the last years. The oral microbiome includes a broad microbial diversity, related with the environment, physiology, and habits of each individual, impacting their health state. In this study, the prevalence of different bacterial groups in young adults with different daily habits was evaluated. The objective was to understand if the prevalence of major bacterial groups are similar, at the phylum or class level, between individuals or, if they differ if it could be associated with specific behaviours. Since the study aimed to quantify specific taxonomic groups, the technic quantitative polymerase chain reaction was used for that purpose (qPCR). The study focused on saliva samples donated by an anonymous group of 51 young adults, dental medicine students, that filled out a questionnaire about different behaviours and daily habits. The participants, after informed of the objectives and type of study, gave their informed consent to the realization of this study. Based on the extraction of total DNA, the abundance of total bacteria and abundance of the phyla *Bacteroidota*, *Bacillota* and *Actinomycetota* and the classes *Gamma*- and *Betaproteobacteria* was assessed. The used primers targeted the 16S rRNA gene, being the sequences specific to different bacterial groups. The abundance of the 16S rRNA gene per volume of sample ranged from 7.3 and 10.0 log ncopies/mL. The phylo *Bacteroidota* was observed as the most abundant – 6.3 to 9.3 log-units ncopies/mL, in contrast to the *Bacillota* that presented a lower abundance between 5.5 and 8.1 log-units ncopies/mL. From a set of 19 variables related to personal characteristics and habits that were organized in a binary scale (present/absent or frequently/unusual), it was observable that the sex, consume of antibiotics in the last 12 months, use of chewing gum, onychophagia and biting of pencils or pens were associated with significantly differences in the abundance of some bacterial groups. These differences were most notorious in the cases of *Bacillota*, *Bacteroidota, Actinomycetota and Gammaproteobacteria* and sex and antibiotic intake, *Betaproteobacteria* and CPO and chewing gum use and other bacterial groups and onychophagia and pen biting. Although it is not possible to establish a relation of cause-effect in these associations, it is possible deduce that some specific habits can contribute to shape the oral cavity microbiota.

Key-words: oral microbiota; qPCR; saliva; *Actinomycetota; Bacteroidota; Bacillota; Pseudomonadota*

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List of Abbreviations and Symbols

NGS	New generation sequencing techniques
T2DM	Diabetes Mellitus Type II
qPCR	Quantitative Polymerase Chain Reaction
FMDUP	Faculdade de Medicina Dentária da Universidade do Porto
CPO	Carious, lost and clogged teeth
bp	Base pairs
DNA	Deoxyribonucleic acid

Introduction

1. Human microbiome and microbiota

In this manuscript, the terms microbiome and microbiota will be used according to the definitions proposed by (Peters et al., 2018)Berg et al., (2020) and Scotti et al., (2017), in which microbiota refers to the variety of microorganisms present in a specific environment, while microbiome also represents the genes, genomes, metabolites, products of the microbiota, as well as the interaction with the respective habitat.

It has been estimated by summing the cell counts for individual organs for a young adult (70 kg and 1.72 meter of height), that the number of human cells present in the body of an adult ranges between 3.0 - 3.7 x 10^{13} cells (Bianconi et al., 2013; Sender et al., 2016). The human body is inhabited by a population of around 1.0 x 10^{14} one hundred trillion bacteria, archaea, fungi, viruses, among other microorganisms, commensal or pathogenic (Cong & Zhang, 2018; Scotti et al., 2017). From these, by calculating an estimation of bacterial cells in each human organ of the average man created by Bianconi et al., (2013) and summing them, Sender et al., (2016), estimated a number of bacterial cells in the human body of approximately 4.0 x 10^{13} cells. Although this number still needs to be verified, microbial cells seem to have at least the same abundance of somatic cells (Gilbert et al., 2018; Sender et al., 2016).

A symbiotic relationship between the microorganisms and the host is established (Cong & Zhang, 2018). While the human body represents a source of nutrients, the microbes have important functions for the host, such as the development and regulation of the immune system, metabolization of molecules that cannot metabolized by the host, outcompete or inhibit pathogens, synthesis of vitamins and affectation of hormones production, aid in nutrition, pH regulation and others (Cong & Zhang, 2018; Gilbert et al., 2018; Scotti et al., 2017).

The first colonization of the human host happens at the moment of birth (Kalbermatter et al., 2021). Depending on the type of childbirth, new-borns acquire microbiota from the vaginal canal and perineum or the mother´s skin in case of a caesarean section (De Luca & Shoenfeld, 2019; Dominguez-Bello et al., 2019). The microbiome development continues after birth, partly due to the ingestion of maternal milk. This milk can shape the microbiota because of its prebiotic and probiotic composition. Some of the compounds present in the milk are human milk oligosaccharides (HMOs) (glycans that babies cannot digest), complex carbohydrates and proteins, end-products of human metabolism, gangliosides, immunoglobulin A (IgA) (De Luca & Shoenfeld, 2019). IgA helps to protect from pathogens while the compounds that cannot be digested by the baby are used by specific microorganisms causing these to proliferate (lactic acid bacteria, *Bifidobacterium* species) (Dominguez-Bello et al., 2019; Reid & Greene, 2014). Changes in these first years happen mainly to the different types of nutrition that the infant experiments. Microbiota will reach a stable state similar to the one from an adult after two years (Reid & Greene, 2014).

The conditions prevailing in differences body regions (e.g. tissues and mucosa, oxygen concentration, moisture, nutrients, functions) lead to the establishment of microbial communities that may be specific of each site, compartment or organ, often differing among individuals(Berg et al., 2020; Reid & Greene, 2014).

1.1. Site-specific microbiota

The Human Microbiome Project (HMP) (https://hmpdacc.org/hmp/), initiated in 2007, consisted of sampling 300 healthy subjects in different body sites with the main objective of characterizing the human microbiome. This major footstep in the knowledge of the human microbiome, possible due to the NGS techniques, triggered the creation of other databases body site-specific. One of these is the Human Oral Microbiome Database (HOMD). Dewhirst et al., (2010) gathered and analysed 16S rRNA genes sequences obtained from associated laboratories for twenty years from different sites of the oral cavity of subjects in different health states or disease states (Palmer, 2014). Nowadays, the database is called expanded Human Oral Microbiome Database (eHOMD) (http://www.ehomd.org), is being curated and is open-access and contains eight hundred 16S rRNA sequences and six hundred and nineteen taxa (Escapa et al., 2018).

The human gut microbiome is the most studied, in part due to the high phylogenetic and functional richness and diversity (Gupta et al., 2017; Reid & Greene, 2014). Examples of critical gut microbiome functions are fat regulation, production and synthesis of short-chain fat acids (SCFA) and vitamins (Cong & Zhang, 2018). The gut microbiome is highly dynamic and strongly influenced by external variables, being influenced by factors such as sleep patterns, geography, age and lifestyle (Cong & Zhang, 2018; Dominguez-Bello et al., 2019; Gupta et al., 2017).

The human body is colonized by a wide diversity of bacteria of various phyla, although is some corporal regions the microbiota is still poorly characterized (Figure 1). Bacterial eco-physiology rule the distribution and function of the human microbiota, as it is shown in Figure 2, adapted from Scotti et al., (2017).

The human microbiome, in a balanced relationship with the host, has an essential role for the health condition and proper physiologic and behavioural development (Chen et al., 2020; Nearing et al., 2020). In contrast, a dysbiosis situation, meaning a "compositional shift in the population of a particular microbial community that promotes development of an inflammatory or disease state" (Baker et al., 2017), may trigger or emerge due to oral and systemic diseases (Ghensi et al., 2020; Graves et al., 2019; Nearing et al., 2020; Radaic & Kapila, 2021).

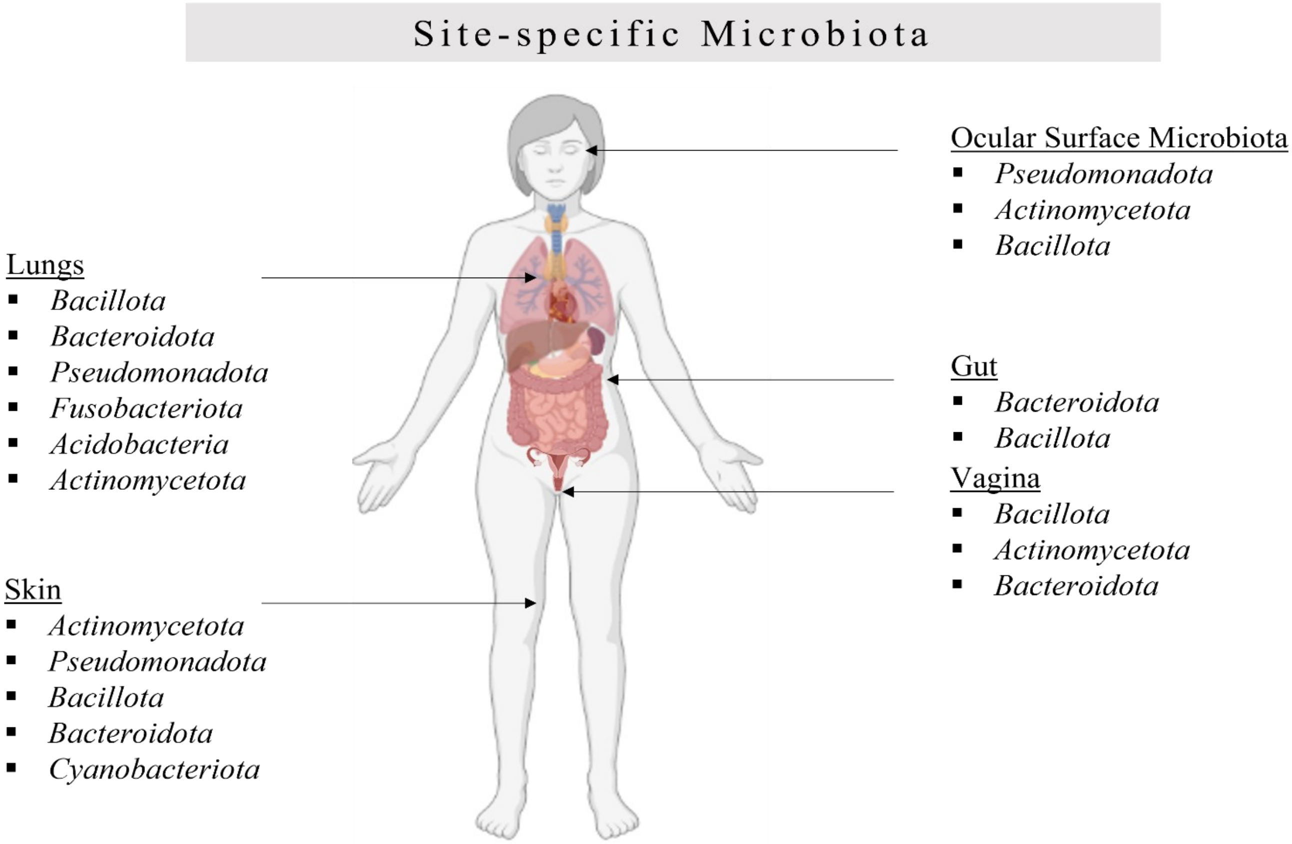


Figure 1 - Human body site-specific microbiota: Different body sites, with different conditions and demands for different functions, present different microbiota. In the figure above is possible to verify the most abundant phyla in certain body-sites: gut (Lloyd-Price et al., 2016); lung (Barcik et al., 2020); ocular (L. J. Lu & Liu, 2020); skin (Zhai et al., 2018) and vagina (Lloyd-Price et al., 2016).

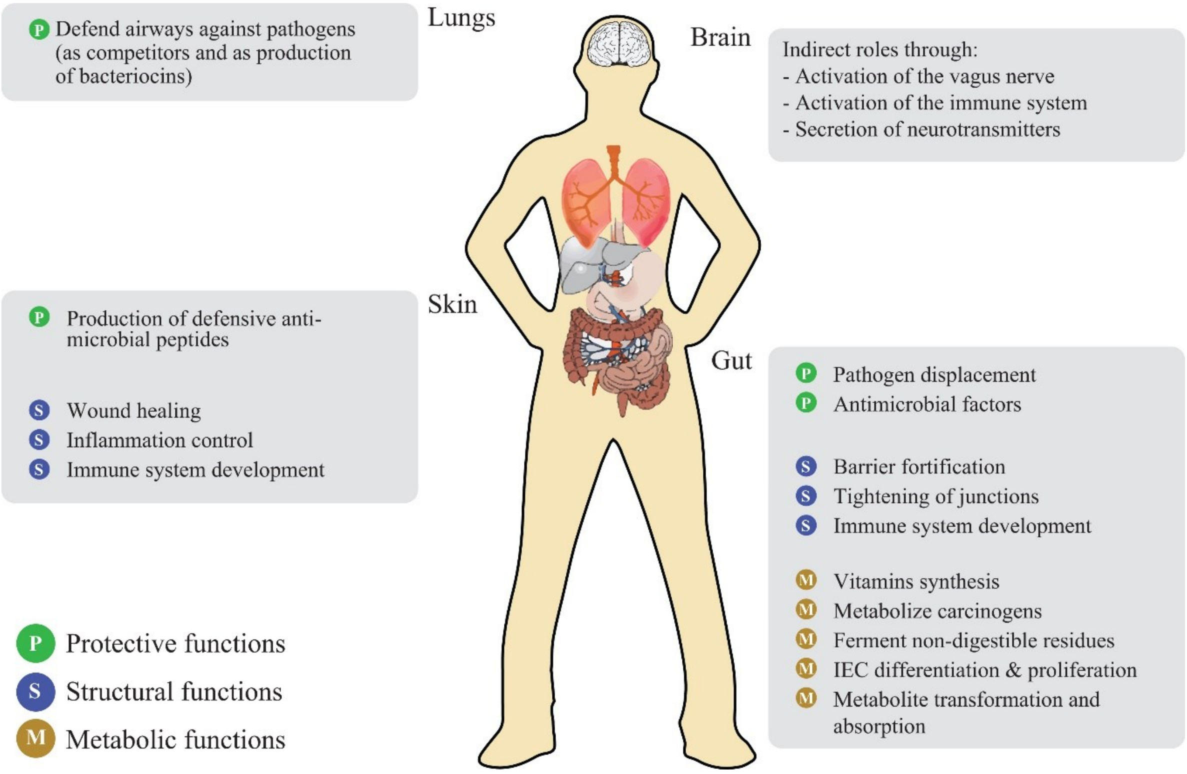


Figure 2 - Main functions of bacteria in the human body – functions performed are divided into three major categories: protective, structural, and metabolic. In the gut, these functions are typically related to the metabolism and metabolization of compounds not metabolized by humans; in the lungs, bacteria aid in allergies inhibition and decrease of excessive immune response (Dominguez-Bello et al., 2019); skin homeostasis is kept by the microbiota (Cong & Zhang, 2018); and vagina microbiota is responsible for balancing the pH and protects against pathogenic microorganisms (Gilbert et al., 2018).

2. Oral Cavity

The human oral cavity represents the most important entry portal for microorganisms (Jenkinson, 2011). Hard and soft tissues (Figure 3) (Deo & Deshmukh, 2019; W. Li & Ma, 2020; Sharma et al., 2018) are colonized, often with a site-specificity distribution. Nevertheless, the same microorganisms can also be observed in different parts of the oral cavity, with variable abundance (Graves et al., 2019; Krishnan et al., 2017; Willis & Gabaldón, 2020). Site-specificity is due to the characteristics of the microhabitats, such as oxygen availability, type of tissue, nutrient acquisition, among others, that influence microorganisms proliferation and/or adherence to surfaces (Krishnan et al., 2017). In general, the oral cavity offers beneficial conditions for the growth of microorganisms, since access to nutrients, water, optimal growth temperature (37°C) are available, with a pH ranging from 6.5 to 7.0, and aerobic and non-aerobic sites (Deo & Deshmukh, 2019; Sharma et al., 2018).

In the first years of the host life, the oral cavity undergoes an age-driven process that leads to alterations in the microbiota composition, due to events like teeth eruption, changes in the type of nutrition, the establishment of the immune system, among other (W. Shi et al., 2018). Throughout life the oral cavity microbiota undergoes successive changes due to diverse intrinsic and external factors (Sharma et al., 2018; Willis & Gabaldón, 2020). These changes may have beneficial implications or adverse consequences to the host (Cornejo Ulloa et al., 2019).

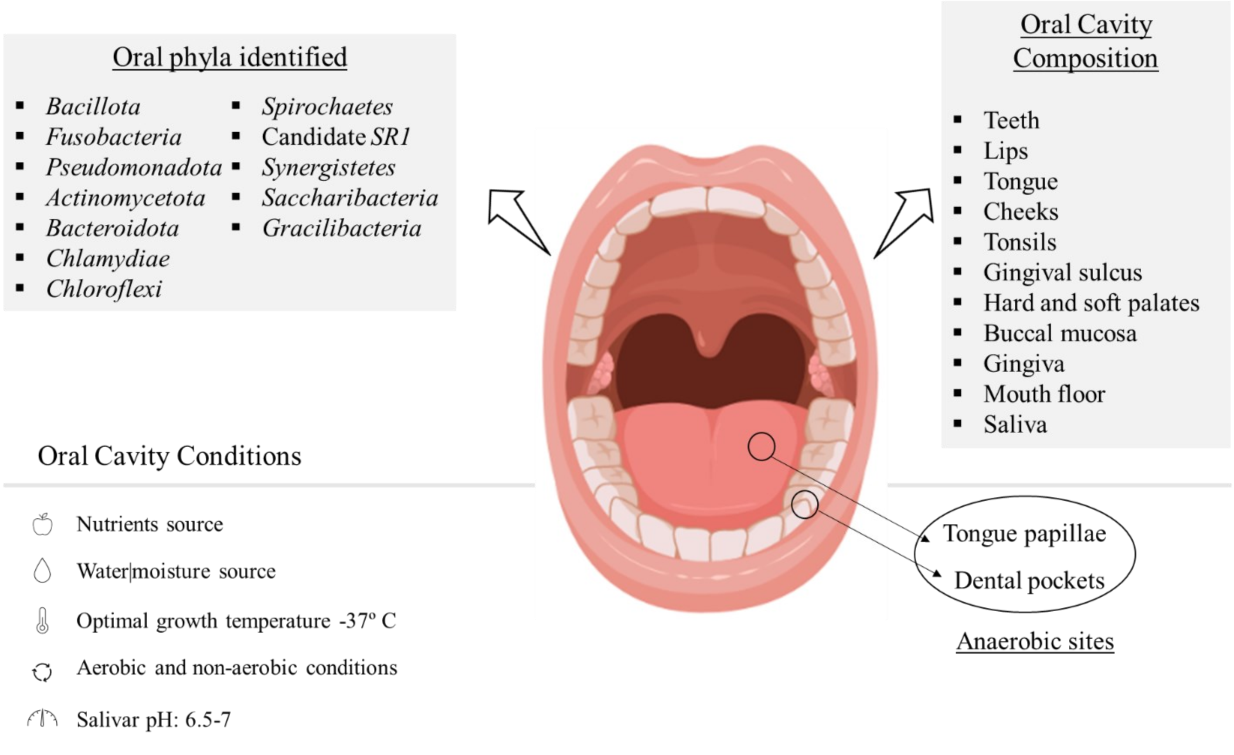


Figure 3 - Oral Cavity: site composition, microorganisms and conditions.

The most important/well studied dysbiosis cases in the oral microbiome are caries, periodontitis and oral cancer (Burcham et al., 2020; Chen et al., 2020). A dysbiosis state is often described based on the comparison with the "normal", usually designated as a healthy state. Nonetheless, it is still difficult to define what characterizes a microbiome of a healthy individual and the boundaries between healthy and dysbiosis conditions (Dong et al., 2018; B. Liu et al., 2012). The oral cavity is connected with other parts of the body, like the middle ear, pharynx, respiratory system, digestive system, and blood due to its high vascularization (Chen et al., 2020; Sharma et al., 2018; Willis & Gabaldón, 2020). As a consequence, in dysbiosis situations of the oral microbiota, other body parts can also be affected, leading to the occurrence of systemic diseases such as obesity, diabetes, inflammatory bowel disease, cardiovascular diseases, adverse pregnancy outcomes, colorectal, pancreatic and liver cancer and autoimmune diseases (Burcham et al., 2020; Chen et al., 2020; M. Lu et al., 2019). More studies are necessary to understand if the alterations verified in the microbiota are what causes the diseases or a secondary effect of the disease development (Li et al., 2019). The hypothesis that the oral microbiota can be used as a biomarker in health diagnosis (Nearing et al., 2020) is highly pertinent, given the simplified access to samples and being a non-invasive and rapid process, it presenting, between subjects from the same geographical locations, lower beta-diversity making comparison between subjects easier, lower costs for sample collection, processing and analysis (Papale et al., 2022; Peng et al., 2022; Stasiewicz & Karpiński, 2022; Zhang et al., 2022)

2.1. Factors influencing the oral microbiome

Oral cavity microbiome can be affected by diet, health condition, age, external environment, and smoking habits, among other factors (Gao et al., 2018a). Since the composition of the oral microbiome undergoes changes, the relative abundance of different bacterial groups is expected to be change throughout the human life span (W. Shi et al., 2018).

Nutrition and the host dietary habits (e.g. maternal milk or solid food; type of food gathering, such as hunter-gathering, farming communities or urban industrial groups, omnivore, vegetarian or vegan) are a major driver of the microbiota composition (Reid & Greene, 2014). As demonstrated in Nasidze et al. (2011), Batwa pygmies, as a hunter-gathering community, thereby diet richer in protein, presented higher diversity of bacteria compared to farming communities. With the transition in lifestyle to a more industrialized type, a decrease in diversity and increasing dominance of bacteria related to caries is also verified (Adler et al., 2014; Gupta et al., 2017). Concerning the type of diet, differences in community structure and beta-diversity were observed when comparing omnivores with vegans (Hansen et al., 2018).

Saliva aids in the colonization and biofilm formation of the oral surfaces (Pedersen & Belstrøm, 2019). Saliva characteristics (e.g. pH, iron and glucose concentration) is also an important factor. For example, glucose consumption may lead to its increase in saliva with a decrease in pH (Goodson et al., (2017). At lower pH, caries-related bacteria can survive and produce lactic acid enabling higher caries incidence (Krishnan et al., 2017;

Zhou et al., 2017). This is also observed in cases of high iron concentration. In cases of obesity or diabetes mellitus type II (T2DM), alterations of saliva characteristics, can help explain the differences verified in the oral cavity microbiota (Goodson et al., 2017).

Smoking habit is another example of factors that can shape the oral cavity microbiota. Wu et al. (2016) collected oral wash samples from the mouth of current and non-current smokers. The most interesting result from this study was that smokers or previously smokers had a depletion of *Pseudomonadota* phylum comparatively to never smokers. As also verified in Sato et al., (2020), microbiome composition differed between smokers and non-smokers. A higher probability of periodontitis incidence is also verified in smokers (Belstrøm et al., 2014).

A large number of other factors can lead to differences in the oral cavity microbiota (Ulloa et al., 2019). Some of these factors, disease states and age, and their relation to the relative abundance of the taxonomic groups in the study, are addressed in the next chapter.

2.2. Oral Cavity Microbiota Composition

The oral cavity holds a diverse microbiota composed of viruses, bacteria and fungi (Jenkinson, 2011; M. Lu et al., 2019). Bacteria is the most studied group in part due to the easiness of cultivation and detection and high prevalence in the human body, including the oral cavity (Baker et al., 2017). More than seven hundred different bacterial taxa have been identified (Abe et al., 2020), being present about one to two hundred different bacteria in "healthy" oral cavity (Willis & Gabaldón, 2020). From the twelve phyla reported by Deo & Deshmukh, (2019) in the oral cavity, the most abundant belong to the *Bacteroidota (17.3% of relative abundance)*, *Bacillota (36.7%)* , *Pseudomonadota (17.1%), Actinomycetota (11.6%)* (Deo & Deshmukh, 2019; Dewhirst et al., 2010; M. Lu et al., 2019). Genera such as *Streptococcus (Bacillota), Actinomyces (Actinomycetota), Bifidobacterium (Actinomycetota), Rothia (Actinomycetota), Moraxella (Pseudomonadota), Neisseria (Pseudomonadota), Veilonella (Bacillota), Campylobacter (Pseudomonadota) , Prevotella (Bacteroidota)* are associated with a healthy host state (Deo & Deshmukh, 2019).

Table 1 summarizes the analysed literature related to the abundances of different taxonomic groups in the oral microbiota and those abundances are organize in table 2.

Table 1 - Information related to the fifteen studies selected for analysis of relative abundance of the phylum in study

Reference	Title	Oral site	Subjects information	Methodology
Bik et al., 2010	Bacterial diversity in the oral cavity of ten healthy individuals	Oral tissues	Individuals with healthy oral tissues	16S rRNA gene amplification and sequencing
Chaudhari et al., 2020	Gut, oral and skin microbiome of Indian patrilineal families reveal perceptible association with age	Oral washing	Healthy, Indian patrilineal families	V3-V4 region of the 16S rRNA gene was done using Illumina Miseq platform
Chen et al., 2018	Oral microbiota dysbiosis and its association with Henoch-Schönlein Purpura in children	Oral swabs / inside cheecks	Henoch-Schönlein Purpura | Healthy controls	16S rRNA gene amplicon sequencing (Illumina) of the V1-V2 hypervariable region
Chen et al., 2020	The oral microbiome profile and biomarker in Chinese type 2 diabetes mellitus patients	Oral swabs / buccal mucosa	T2DM subjects | Healthy controls	Amplification of V1-V2 region of the 16S rRNA gene and Illumina sequencing
Dewhirst et al., 2010	The Human Oral Microbiome	Oral isolates	Different oral health and diseases stages	16S rRNA gene amplification and Sequencing
Dong et al., 2018	Microbial Similarity and Preference for Specific Sites in Healthy Oral Cavity and Esophagus	Tongue / Supragingival plaque / Saliva	Healthy individuals	Amplification of the V3-V4 region of the 16S rRNA gene and Illumina sequencing
Jiang et al., 2019	The Oral Microbiome in the Elderly With Dental Caries and Health	Plaque samples	Patients with caries and healthy controls; Only caries-free results	V3-V4 region of the 16S rRNA gene was done using Illumina Miseq platform
Keijser et al., 2008	Pyrosequencing Analysis of the Oral Microflora of Healthy Adults	Saliva / Supragingival plaque	Healthy adults	V6 hypervariable region by Pyrosequencing
Ren et al., 2017	Exploring the oral microflora of preschool children	Supragingival plaque / Saliva / Tongue dorsum	Children	PCR amplification of V1–V3 hypervariable regions of 16S rRNA and Pyrosequencing
Schulz et al., 2019	Comparison of the oral microbiome of patients with generalized aggressive periodontitis and periodontitis-free subjects	Teeth pockets	Agressive periodontitis | Periodontitis free	PCR amplification of theV3-V4 region of the 16S rRNA for Illumina sequencing
Snider et al., 2018	Barrett’s esophagus is associated with a distinct oral microbiome	Saliva / Buccal Lining / Tongue dorsum / Hard palate / superior labial funulum	Barrett´s esophagus disease | Healthy controls	Sequencing of the V4 region of the 16S rRNA gene by Illumina
Topcuoglu et al., 2019	Oral microbial dysbiosis in patients with Kostmann syndrome	Whole saliva samples	Kostmann syndrome | Healthy controls	PCR amplification of the V3-V4 region of the bacterial 16S rRNA gene and sequencing by Illumina
Verma et al., 2020	Taxonomic profiling and functional characterization of the healthy human oral bacterial microbiome from the north Indian urban sub-population	Mouthwash	Healthy north indian urban sub-population	PCR amplification of the V3 region of the 16S rRNA for Illumina sequencing
Zhao et al., 2020	The dynamics of the oral microbiome and oral health among patients receiving clear aligner orthodontic treatment	Unstimulated whole saliva	Before invisalign treatment | 6 months after first invisalign treatment	Amplification of the V3-V4 region of the 16S rRNA gene and Illumina sequencing
Zhou et al., 2018	Oral microbial flora of patients with Sicca syndrome	Saliva	Sicca syndrome | Healthy controls	Amplification of the V4-V5 region of the 16S rRNA gene + Sequencing (Illumina)

Table 2 - Relative abundance of Bacillota, Actinomycetota, Gammaproteobacteria, Pseudomonadota and Bacteroidota

Reference	Sample size	Number of clones/NGS reads	Relative Abundance (%)				
			Bacillota	*Actinomycetota*	*Gammaproteobacteria*	*Pseudomonadota*	*Bacteroidota*
Bik et al., 2010	10	11368 sequences	33,2	14,5	not analysed	27,5	16,6
Dewhirst et al., 2010	1000	36043 clones	36,7	11,6	not analysed	17,1	17,3
Jiang et al., 2019	22	1740207 sequences	29,6	7,6	not analysed	20,4	22,4
Dong et al., 2018	27	not mentioned	14,48	9,26	4,97	not analysed	32,2
Chen et al., 2020	280	not mentioned	23,51	not analysed	not analysed	63,46	6,5
Chen et al., 2020	162	not mentioned	14,48	not analysed	not analysed	71,56	11,41
Zhao et al., 2020	25	1853952 reads	37,08	7,0	not analysed	21,32	21,47
Zhao et al., 2020	25	1853952 reads	39,26	8,59	not analysed	26,11	15,74
Snider et al., 2018	32	30103626 contigs	27,1	10,3	not analysed	23,8	30,3
Snider et al., 2018	17	30103626 contigs	14,6	12,2	not analysed	34,5	29,6
Topcuoglu et al., 2019	9	2170895 sequences	62	8	not analysed	10	10
Topcuoglu et al., 2019	9	2170895 sequences	42	10	not analysed	20	15
Ren et al., 2017*	10	314639 reads	0,18	0,22	not analysed	not analysed	0,2
Ren et al., 2017*	10	314639 reads	0,54	0,1	not analysed	not analysed	0,1
Ren et al., 2017	10	314639 reads	0,4	0,13	not analysed	not analysed	0,12
Chen et al., 2018	98	not mentioned	40,75	3	not analysed	25,99	23,97
Chen et al., 2018	66	not mentioned	34,81	1	not analysed	50,44	9,92
Zhou et al., 2018	9	535846 sequences	34,08	not analysed	not analysed	16,51	35,63
Zhou et al., 2018	5	535846 sequences	28,02	not analysed	not analysed	42,95	18,82
Schulz et al., 2019	13	17000000 sequences	10	<10	not analysed	<10	45
Schulz et al., 2019	13	17000000 sequences	20	1	not analysed	42	20
Verma et al., 2020	20	6254320 reads	28,87	4,14	not analysed	25,17	16,69
Chaudhari et al., 2020	54	7568649	24	2	not analysed	34	32
Keijser et al., 2008	71	197,600 sequences	40,7	6,3	not analysed	21	27.2
Keijser et al., 2008	98	197,600 sequences	32,4	25	not analysed	16	14.9

Concerning the classes of the *Pseudomonadota* phylum, the relative abundance of each was reported, in healthy individuals, as follow: *Betaproteobacteria* – 29%; *Gammaproteobacteria* – 5.0%; *Epsilonproteobacteria* – 1.2%; *Alphaproteobacteria* – 0.37% and *Deltaproteobacteria* – 0.11% (Dong et al., 2018). Most of the studies, depending on the specific objectives, do not analyse the abundance of each class but rather the abundance of a particular one, such as genera or species. For example, *Neisseria* and *Haemophilus* have been determined as the most abundant genera of the *Pseudomonadota* phylum in many studies (Bik et al., 2010; Jiang et al., 2019; Zaura et al., 2009). However, *Cardiobacterium* and *Campylobacter* were also associated with the microbiome of the oral cavity from healthy individuals (Bik et al., 2010).

Pseudomonadota have been reported at variable proportions, ranging from 16% up to 72%. Although these results may depend on the methods used (16S rRNA sequencing and metagenomics – Illumina sequencing, Table 1), unveil that the oral microbiota is rich in *Pseudomonadota*, possibly adsorbed, as the abundances found by Zhao *et al.*, Keijser *et al.*, Topcuoglu *et al.* and Verma *et al.* for this phylum are similar for the healthy subjects (10-26.11%) and can be explained by the fact that the studies Keijser et al., (2008) , Topcuoglu et al., (2019) and Zhao et al., (2020) collected similar type of samples (saliva and supragingival plaque, whole saliva, unstimulated saliva, respectively), and that Topcuoglu et al., (2019), Verma et al., (2020) and Zhao et al., (2020) present the same methodology (amplification of the V3-V4 region of the 16S rRNA gene and Illumina sequencing). Highest abundance in healthy individuals (72% and 50%) was reported when oral swab was the collecting method (Chen et al., 2018, 2020).

Members of the phylum *Bacillota* have been reported in percentages ranging from 14% to 41 % (Chen et al., 2018; Dong et al., 2018; Snider et al., 2018). *Bacteroidota* have been reported to vary between 10% and 32.%.

Actinomycetota is, in most of the analysed articles, observed as the phylum with lower abundance in the oral cavity. Its values range between 1% up to 25%. In the study with the highest abundance, the phylum concerned was the second most abundant and might be explained by the oral site of the collected samples, different from all the others, the supragingival plaque (Keijser, Zaura, Huse, van der Vossen, et al., 2008).

Being the connecting point to the interior of our organism and being exposed to the environment, the oral cavity microbiome importance to the host health is still to be completely understood (Willis & Gabaldón, 2020). Currently, studies already showed the existence of a relationship between the oral microbiome and oral and systemic diseases (periodontitis, oral and pancreatic cancer, T2DM, Crohn´s disease, obesity, among others) (Krishnan et al., 2017; M. Lu et al., 2019). New studies are also important due to new information that might arise from them and help in services like personalized oral care, geriatric health and in providing more life quality for elderly individuals (Gao et al., 2018a).

In conclusion, it is possible to verify differences between microbiomes of each individual, between each oral cavity site and in the presence of health disorders, oral or systemic. As stated more studies are also still required to fully understand what type of relationship exists between the oral microbiomes and disorders.

3. Culture-based and culture-independent methods

Culture-dependent methods are based on culture and isolation of microorganisms using selective media but can also be applied in the determination of the minimum inhibitory concentrations (MIC) (Kisand & Wikner, 2003). Culture of samples in different culture media (selective, differential), microscopy analysis of colonies, cell staining, biochemical tests, growth conditions, nutrients needs, antibiotic resistance (Deo & Deshmukh, 2019) are some examples of this type of methods. Although the advantage of enabling a simple morphologic and physiologic characterization of the microorganisms, the principal disadvantage is the fact that not all microorganisms are culturable or cannot grow due to the impossibility of simulate the characteristics of their environment in a laboratory (Verma et al., 2018). Culture-independent techniques focus on the analysis of the nucleic acids (DNA, RNA), proteins and the whole community (Deo & Deshmukh, 2019). qPCR and DNA/RNA sequencing are examples of these techniques. The presence and abundance of bacteria in different samples can be detected and quantified by quantitative Polymerase Chain Reaction (qPCR) amplification of regions of the 16S rRNA gene or specific target genes. The 16S rRNA gene has a sequence of 1.500 base pairs (bp), and it is highly conserved, being present in all bacteria. This amplification technique gives mainly taxonomic information (Soriano-Lerma et al., 2020). Using specific primers for target genes, it is also possible using this technique to detect and quantified specific genes/ target regions, such as antibiotic resistance genes (Manaia, 2022).

Table 3 summarizes different methods that can be used for the analysis of microbiota and the respective strengths and pitfalls. While there is no perfect method, it is possible to select the most adequate for reach specific goals. The choice of techniques must be done considering sample size, study objectives and sample type (Martellacci et al., 2019; Yan et al., 2020). The Human Microbiome Project (HMP) (https://hmpdacc.org/hmp/), and later the sibling project of the Human Oral Microbiome Database (HOMD), relied mostly 16S rRNA gene amplicon sequencing (Dewhirst et al., (2010) (Palmer, 2014). The later database complied data relative to twenty years of oral cavity samples of individuals with different health conditions. Nowadays, the database is called expanded Human Oral Microbiome Database (eHOMD) (http://www.ehomd.org), is being curated and is open-access and contains eight hundred 16S rRNA sequences and six hundred and nineteen taxa (Escapa et al., 2018). With the reduction of sequencing costs, nowadays, metagenomics is becoming the preferred approach for studying microbiomes (Carda-Diéguez et al., 2022; Gehrig et al., 2022; Y. ; Shi et al., 2022; Walker et al., 2022). Quantitative PCR may be preferred when the search of some minority groups, the determination of absolute quantifications or the search for specific genes, such as the search for antibiotic resistance, is aimed.

Table 3 -Summary of culture-dependent and culture-independent techniques for analysing the oral microbiota (Deo & Deshmukh, 2019; Lagier et al., 2018; Lazarevic et al., 2012; Manaia, 2022; Martellacci et al., 2019; Yan et al., 2020)

Techniques	Example of Techniques	Advantages	Disadvantages
Culture-dependent	Selective and differential culture media Cell´s staining Microscopy Biochemical Tests Antibiotic susceptibility	Phenotypic, morphologic and physiologic characterization of microorganisms	Requires more laboriousness Difficulty on cultivation of certain microorganisms Lengthy process
Culture-independent	qPCR	Affordable Faster Adequate for a small number of targets and detection of previously known sequences	Possibility of incorrect primer use/design High limit of quantification Limited information
	16S rRNA-amplicon sequencing	Lower costs Less computational requirements Large number of samples	Less effective for distinguish species Extraction bias
	Metagenomics	Gives more detailed information Requires less quantity of amplified product Large number of samples	Cannot distinguish between dead or alive organisms Bioinformatic bias Samples processing bias

4. Bacterial taxonomic groups of interest

Considering the existence and high abundance of certain bacterial phyla in the human body and oral cavity, the study performed focused on four of the major phyla: *Actinomycetota*, *Bacteroidota*, *Bacillota* and *Pseudomonadota* (*Betaproteobacteria* and *Gammaproteobacteria*) (Table 2)

Table 4 – Phyla in study characteristcs

Phylum	Characteristics	References
Actinomycetota	Ubiquitous phylum Aerobic / Anaerobic Gram-positive Non motile No capsule or spore formation Production of antibiotics and bioactive compounds	(Azman et al., 2019) (Lewin et al., 2016) (Fatahi-Bafghi, 2019)
Bacillota	Aerobic / Anaerobic Gram-positive Endospore-forming Synthesis of enzymes and antibiotics Abundant in the gut	(Seong et al., 2018) (Boundless, 2017) (Scotti et al., 2017)
Bacteroidota	Gram-negative Gliding motility Non spore forming Degradation of organic matter Establishes mutualistic relationships Present in saliva and plaque	(Munoz et al., 2020) (Thomas et al., 2011) (Lapébie et al., 2019) (Hoyles & McCartney, 2009)
Pseudomonadota	Facultative or obligatory anaerobes Gram-negative Presents motility Ubiquitous in nature Present in human oral cavity Pathogenic species	(Rizzatti et al., 2017) (Shin et al., 2015) (Rudi et al., 2010)

5. Hypothesis and Objectives

The study was planned to test the hypothesis that the profile, *i.e.* the relative proportions of the major taxonomic groups thriving in the oral cavity may be differ among individuals and that such differences can be associated with distinct intrinsic factors and quotidian habits. The specific stages were proposed:

- To design a qPCR-based protocol that permitted the taxonomic profiling of saliva;
- To characterize the saliva microbiota of a group of young adults, who also reported some habits and characteristics;
- To infer about potential relationships between the measured taxonomic groups and the variables reported by the study participants;

Material and Methods

Samples and individuals´ characterization

The donors of the saliva samples analysed in this study were students of the dental medicine integrated master’s degree at University of Porto. The age of the 51 students enrolled in the study ranged from 18 to 33 years old. The students provided their informed consent to participate in the study and responded a short questionary (Figure 4). The questionnaire was designed considering relevant aspects related to the oral cavity and factors concerning different lifestyles – e.g. age, sex, oral hygiene and health and diet, type of childbirth, contraceptive intake, and oral contact with partners.

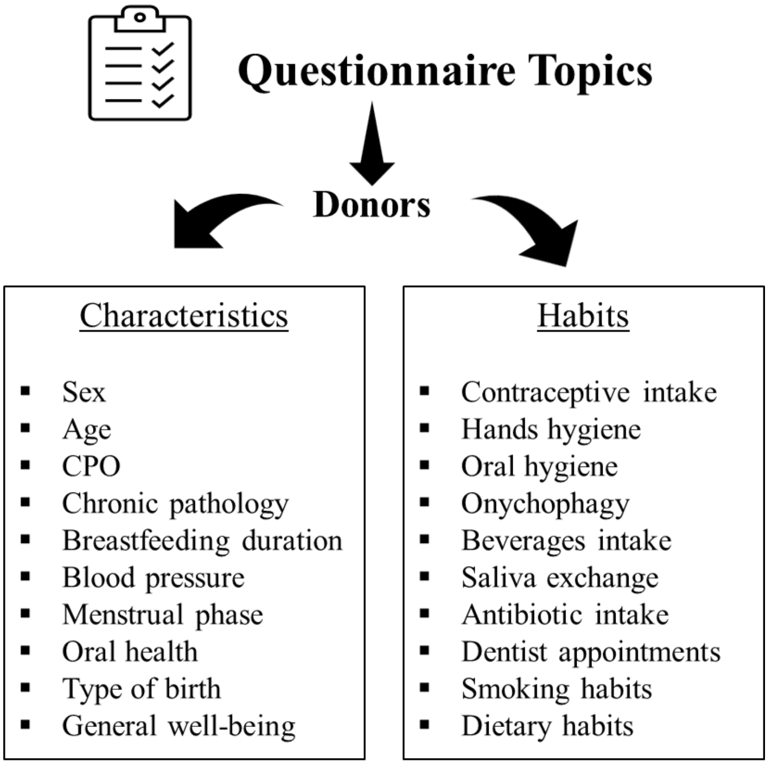


Figure 4 - Questionnaire main topics – all the questions made to the subjects concerned the topics mentioned in the table. For example, in the topic “Dentist appointments”, questions such as dentist appointments regularity, and tartar removal were asked. Carious, lost and clogged teeth (CPO) index was also asked

Samples, consisting of accumulating saliva, were collected after the lunch period, from 2.00 to 6.00 pm. Preparation for sample collection, recommended that no food intake and oral hygiene at least in the two previous hours, and the rinse of the oral cavity with distilled water prior to sampling. Saliva accumulating it in the mouth for five minutes, while swallowing was inhibited, was spat into a 50 mL falcon tube, with an anonymized reference. After measuring the pH and volume, samples were immediately stored at -80°C for further studies.

DNA extraction

The saliva samples were centrifuged at 1 000 x g for 35 minutes (4ºC) to concentrate the biomass. Sediments were resuspended in 600 µL of Phosphate Buffered Saline solution and the total DNA was extracted using the NZY Tissue gDNA Isolation Kit (Nzytech, Lisbon, Portugal), according to manufacturer instructions. Briefly, the homogenised (quick vortex) biomass suspensions were transferred to 2 mL centrifuge tubes containing 25 µL of the Proteinase K (PK) solution and incubated at 56ºC for 10 minutes. To the solution 600 µL of buffer NL was then added followed for a quick vortex and an incubation period of 10 min at 70 ºC. The extracted DNA was then precipitated with 96% ethanol. The samples were then transferred to NZYSpin Tissue columns and the silica membrane was washed by adding Buffer NW1 and NW2 to the column followed by a dry step that consisted in a centrifugation for 2 minutes at 11000 x g. After elution with buffer NE preheated at 70ºC, DNA concentration was quantified using Qubit® 2.0 Fluorometer with the kit Qubit® dsDNA HS Assay (Invitrogen, Carlsbad, CA, USA). DNA extracts were stored at -20°C.

The extraction of DNA from bacterial cultures that would be used as reference DNA for the calibration curves was performed using commercial kits. For *Escherichia coli* was used the QIAamp® Fast DNA Stool Mini kit (QIAgen, Hilden, Germany) and for the other strains, the NZY Tissue gDNA Isolation Kit (Nzytech, Lisbon, Portugal) was used. For the latter kit, before the DNA extraction, a loop of culture was collected to a 2mL eppendorf tube and 180 µL Buffer NT1 and 25µL of PK were added. To obtain higher concentration of DNA from *Peribacillus muralis* R-8192, treatment with lysozyme was done previously to the extraction procedure. It consisted in incubation with the enzyme solution (10mg/mL) for 1 hour at 37ºC. The manufacturers standard protocol for extraction of bacterial cells was followed. For the NZY Tissue gDNA Isolation Kit the samples were incubated for three hours in a water bath boiler at 56ºC, addition of 10 µL RNAse and then the next steps of the manufacturers protocol were followed. After the elution made with 100 µL of pre-heated NE buffer, DNA concentration was determined, with the kit Qubit® dsDNA HS Assay in Qubit® equipment (Invitrogen, Carlsbad, CA, USA) and the samples were stored at -20°C. DNA extracts were preserved up to its use in qPCR assays. The DNA concentrations obtained ranged between 30.00 µg/mL and 53.80 µg/mL.

Primer design and qPCR protocols

Considering the objectives of the study, novel protocols for the quantification of different taxonomic groups - *Actinomycetota*, *Bacillota*, *Bacteroidota*, *Betaproteobacteria* and *Gammaproteobacteria*, were established. The first step was to select the qPCR primers. With this aim, the database Pubmed was searched based on the terms "phylum specific primers" as search sentence. Sorted by best match, 215 results of papers were listed. Twelve of these presented at least one primer set for the taxonomic groups of interest, applied in conventional PCR or qPCR. These papers and the relevant references were also analysed, leading to the inspections of twenty research articles.

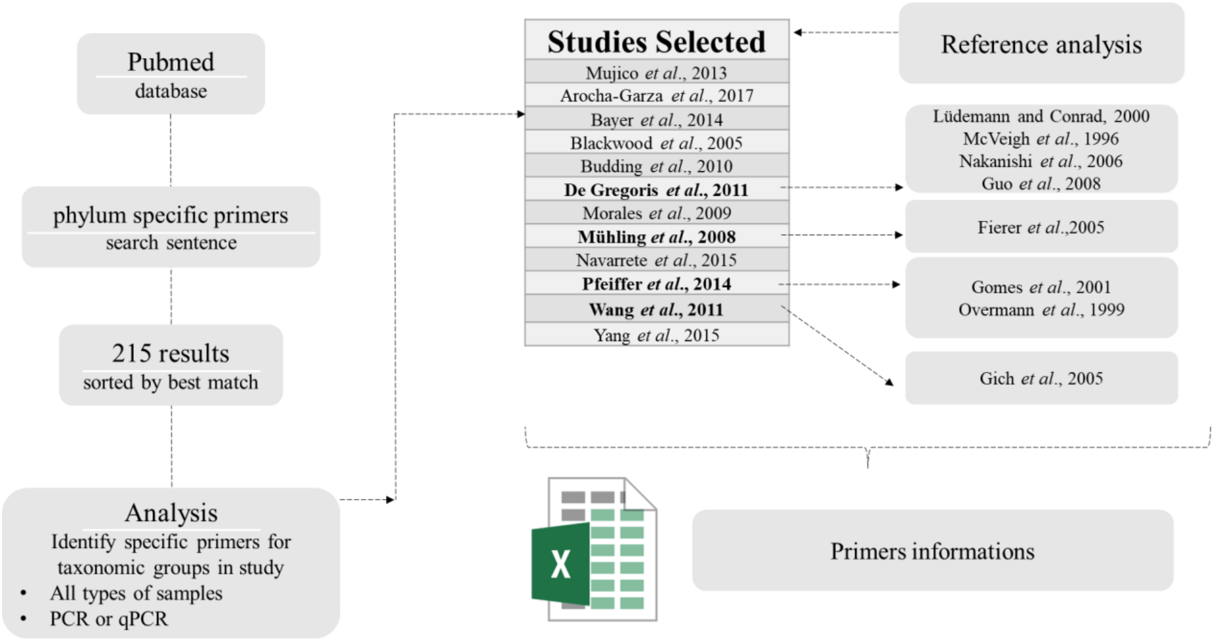


Figure 5 – Bibliographic primers search summary

Primer testing *in silico*

Possible adequate primers selected from the literature were tested *in silico*. Thirty-four primer pairs were selected for these tests (Table 5)

Table 5 - Primers list for the target taxonomic groups retrieved from the analysed literature.

Taxonomic Group	Sample	Reference	Primer set	Primer	Sequence 5´→ 3´	Degenerated Nucleotides
Betaproteobacteria	Human feces and rhizosphere	Pfeiffer et al., 2014	1	Beta359f	GGGGAATTTTGGACAATGGG	0
		Pfeiffer et al., 2014		Beta682r	ACGCATTTCACTGCTACACG	0
	Mouse feces	Yang et al., 2015	2	Beta979F	AACGCGAAAAACCTTACCTACC	0
		Yang et al., 2015		Beta1130R	TGCCCTTTCGTAGCAACTAGTG	0
	Human feces and rhizosphere	Overmann et al., 1999	3	Bet680	TCACTGCTACACGYG	1
		Gomes et al., 2001		F948β	CGCACAAGCGGTGGAT	0
	Soil	Fierer et al., 2005	4	Eub338F	ACTCCTACGGGAGGCAGCAG	0
		Fierer et al., 2005		Bet680R	TCACTGCTACACGYG	1
	Human feces and rhizosphere	Pfeiffer et al., 2014	5	S-C-bProt-0972-a-S-18	CGAARAACCTTACCYACC	2
		Pfeiffer et al., 2014		S-C-bProt-1221-a-A-17	GTATGACGTGTGWAGCC	1
	Soil	Morales et al., 2009	6	715-733fBP	AAYACCRATGGCGAAGGC	2
		Morales et al., 2009		907r	CCGTCAATTCMTTTRAGTTT	2
Gammaproteobacteria	Marine biofilms	De Gregoris et al., 2011	7	1080γF	TCGTCAGCTCGTGTYGTGA	1
		De Gregoris et al., 2011		γ1202R	CGTAAGGGCCATGATG	0
		De Gregoris et al., 2011	8	Gamma395f	CMATGCCGCGTGTGTGAA	1
		De Gregoris et al., 2011		Gamma871r	ACTCCCCAGGCGGTCDACTTA	1
	Mouse feces	Yang et al., 2015	9	Gamma877F	GCTAACGCATTAAGTRYCCCG	2
		Yang et al., 2015		Gamma1066R	GCCATGCRGCACCTGTCT	1
	Soil	Morales et al., 2009	10	680-698fGP	CMKGTGTAGCRGTGAAAT	3
		Morales et al., 2009		907r	CCGTCAATTCMTTTRAGTTT	1

Taxonomic Group	**Sample**	**Reference**	**Primer set**	**Primer**	**Sequence 5´→ 3´**	**Degenerated Nucleotides**
Actinomycetota	Water and upper layer sediment	Arocha-Garza et al., 2017	11	S-C-Act-235-a-S-20	CGCGGCCTATCAGCTTGTTG	0
	Water and upper layer sediment	Arocha-Garza et al., 2017		23SR	AGGCATCCACCGTGCGCCCT	0
	Soil	Fierer et al., 2005	12	Actino235F	CGCGGCCTATCAGCTTGTTG	0
	Soil	Fierer et al., 2005		Eub518R	ATTACCGCGGCTGCTGG	0
	Mouse feces	Yang et al., 2015	13	Act664F	TGTAGCGGTGGAATGCGC	0
	Mouse feces	Yang et al., 2015		Act941R	AATTAAGCCACATGCTCCGCT	0
	Human feces and rhizosphere	Pfeiffer et al., 2014	14	S-P-Acti-1339-a-A-18	TCWGCGATTACTAGCGAC	1
	Human feces and rhizosphere	Pfeiffer et al., 2014		Act920F3	TACGGCCGCAAGGCTA	0
	Human feces and rhizosphere	Pfeiffer et al., 2014	15	Act1200R	TCRTCCCCACCTTCCTCCG	1
	Human feces and rhizosphere	Pfeiffer et al., 2014		S-C-Act-235-a-S-20	CGCGGCCTATCAGCTTGTTG	0
	Marine biofilms	De Gregoris et al., 2011	16	Act920F3	TACGGCCGCAAGGCTA	0
	Marine biofilms	De Gregoris et al., 2011		Act1200R	TCRTCCCCACCTTCCTCCG	1
	Mediterranean sponges	Bayer et al., 2014	17	AB1165f	GTYAACTCGGAGGAAGGT	1
	Mediterranean sponges	Bayer et al., 2014		Act1360r	CTGATCTGCGATTACTAGCGACTCC	0
	Human feces and rhizosphere	Pfeiffer et al., 2014	18	S-P-Acti-0927-a-S-17	GGRCCCGCACAAGCGGC	1
	Human feces and rhizosphere	Pfeiffer et al., 2014		S-P-Acti-1154-a-S-19	GADACYGCCGGGGTYAACT	3
	Soil	Morales et al., 2009	19	697-715fAT	TGCGCAGAKATCRGGARG	3

Taxonomic Group	Sample	Reference	Primer set	Primer	Sequence 5´→ 3´	Degenerated Nucleotides
Actinomycetota	Soil	Morales et al., 2009		907r	CCGTCAATTCMTTTRAGTTT	2
	Italian rice field soil	Lüdemann and Conrad, 2000	20	27f	AGAGTTTGATCCTGGCTCAG	0
	Italian rice field soil	Lüdemann and Conrad, 2000		ABl165r	ACCTTCCTCCGAGTTRAC	1
Bacillota	Human feces and rhizosphere	Pfeiffer et al., 2014	21	S-P-Firm-0352-a-S-18	CAGCAGTAGGGAATCTTC	0
	Human feces and rhizosphere	Pfeiffer et al., 2014		S-P-Firm-0525-a-A-18	ACCTACGTATTACCGCGG	0
	Soil	Fierer et al., 2005	22	Lgc353F	GCA GTA GGG AAT CTT CCG	0
	Soil	Fierer et al., 2005		Eub518R	ATT ACC GCG GCT GCT GG	0
	Mice feces	Mujico et al., 2013	23		GGAGYATGTGGTTTAATTCGAAGCA	1
	Mice feces	Mujico et al., 2013			AGCTGACGACAACCATGCAC	0
	Mouse feces	Yang et al., 2015	24	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	1
	Mouse feces	Yang et al., 2015		Firm1060R	AGCTGACGACAACCATGCAC	0
	Marine biofilms	De Gregoris et al., 2011	25	928F-Firm	TGAAACTYAAAGGAATTGACG	1
	Marine biofilms	De Gregoris et al., 2011		1040FirmR	ACCATGCACCACCTGTC	0
	Marine biofilms	De Gregoris et al., 2011	26	Firm350f	GGCAGCAGTRGGGAATCTTC	1
	Marine biofilms	De Gregoris et al., 2011		Firm814r	ACACYTAGYACTCATCGTTT	2
Bacteroidota	Marine Biofilms	De Gregoris et al., 2011	27	MIBF	GGCGACCGGCGCACGGG	0

	Marine Biofilms	De Gregoris et al., 2011		MIBR	GRCCTTCCTCTCAGAACCC	1

Taxonomic Group	**Sample**	**Reference**	**Primer set**	**Primer**	**Sequence 5´→ 3´**	**Degenerated Nucleotides**
Bacteroidota	Marine Biofilms	De Gregoris et al., 2011	28	798cfbF	CRAACAGGATTAGATACCCT	1
	Marine Biofilms	De Gregoris et al., 2011		cfb967R	GGTAAGGTTCCTCGCGTAT	0
	Human feces and rhizosphere	Pfeiffer et al., 2014	29	S-P-Bdet-0107-a-S-21	GCACGGGTGMGTAACRCGTAT	2
	Human feces and rhizosphere	Pfeiffer et al., 2014		S-P-Bdet-0309-a-A-21	GTRTCTCAGTDCCARTGTGGG	3
	Soil	Morales et al., 2009	30	685-703BT	GTAGCGGTGAAATGCWTA	1
	Soil	Morales et al., 2009		907r	CCGTCAATTCMTTTRAGTTT	2
	Mice feces	Mujico et al., 2013	31		GGARCATGTGGTTTAATTCGATGAT	1
	Mice feces	Mujico et al., 2013			AGCTGACGACAACCATGCAG	0
	Soil	Fierer et al., 2005	32	Cfb319	GTA CTG AGA CAC GGA CCA	0
	Soil	Fierer et al., 2005		Eub518R	ATT ACC GCG GCT GCT GG	0
	Mouse feces	Yang et al., 2015	33	Bac960F	GTTTAATTCGATGATACGCGAG	0
	Mouse feces	Yang et al., 2015		Bac1100R	TTAASCCGACACCTCACGG	1
	Pig feces	Guo et al., 2008	34	Bact934F	GGARCATGTGGTTTAATTCGATGAT	1
	Pig feces	Guo et al., 2008		Bact1060R	AGCTGACGACAACCATGCAG	0

The tests consisted on Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) search (Ye et al., 2012). Nucleotide sequences of forward and reverse primers were submitted in the 5´-> 3´ orientation. The parameters (e.g. amplicon size, melting temperatures of primers, , primer specificity stringency, maximum target amplicon size) were left as default except for the “database” and “organism”. In the output data, the GeneBank accession number, species, taxonomic group to which belongs, product length in base pairs (bp) and the number of total products were registered for the first fifteen results for each tested primer pair.

Choosing a pair of primers for each of the taxonomic groups of interest was done evaluating the results obtained from Primer Blast and comparing their characteristics with the desirable ones of qPCR primers found in the literature (KAPABYOSISTEMS, 2017; Technologies, 2013). The selection criteria were specificity – the primers only targeted the taxonomic group of interest; a single region of the target gene base-paired with the primers pairs; the molar guanine plus cytosine content was below 65% and predicted amplicons are < 400 bp (Chuang et al., 2013; Quellhorst & Rulli, 2012).

A primer pair was selected for the phyla *Bacteroidota, Bacillota, Actinomycetota,* or the classes *Gammaproteobacteria* and *Betaproteobacteria.* The selected primers were further verified for these groups through Primer Blast and analysis of the first one hundred results.

Table 6 – qPCR Primers´ criteria for inclusion or exclusion – GC – molar guanine plus cytosine content

qPCR Primers	
Inclusion criteria	**Exclusion criteria**
GC < 65%	Amplification of other taxonomic groups
Amplification of expected taxonomic group	Appears more than once
Amplicon size < 400 bp	Amplification products with different size
Only 1 amplification product	

Primers´ specificity was later tested by PCR reactions with reference strains representative of each target group.

Reference-strains

The reference strains were *Escherichia coli* (ATCC 25922) (Minogue et al., 2014), for *Gammaproteobacteria*, *Bordetella petrii* (DSM 12804) (von Wintzingerode et al., 2001) for *Betaproteobacteria*, *Peribacillus muralis* R-8192 (DSM 16288, renamed from *Bacillus muralis*) (Kuisiene et al., 2008; Patel & Gupta, 2020) for *Bacillota*, *Olivibacter domesticus* (CCUG 54353, renamed from *Pseudosphingobium domesticum*) (Siddiqi et al., 2018; Vaz-Moreira et al., 2007) for *Bacteroidota* and *Humibacter albus* SC-083 (DSM 18994) (Vaz-Moreira et al., 2008) for *Actinomycetota*. The cultures were available in the laboratory culture collection, stored at -80ºC and were cultured on the culture medium Plate Count Agar (PCA) (Liofilchem®, Italy) at 30°C during 24 h. Purity was verified based on colony and cell morphology.

Genome size and number of copies of the 16S rRNA gene per genome are required to correctly calculate the number of gene copies in the references used for the qPCR. For that reason, the genome of each standard strain, assembly level, genome size and the number of 16S rRNA gene copies were verified. All the strains presented an assembly at the "complete genome level" except *Humibacter albus* and *Olivibacter domesticus*, having the assembly at a "contig" and "scaffold" level, respectively. Since the representation of the species genome was classified as "full" for both, the number of copies of the 16S rRNA gene was verified using the tool, ContEst16S (I. Lee et al., 2017) from EZBioCloud (Chunlab Inc, Seoul, Korea).

The assembled genomes of *Humibacter albus* strain XXX and *Olivibacter domesticus* strain XXX were downloaded from NCBI ("Download assembly"-"Genbank" as source – file in "Genomic FASTA") respectively (https://www.ncbi.nlm.nih.gov/assembly/GCF_000421825.1 , https://www.ncbi.nlm.nih.gov/assembly/GCF_900109575.1/). Entering the EZBioCloud site (https://www.ezbiocloud.net/tools/contest16s), each file was uploaded and run in the tool. Considering that the possible variation in copies of 16S rRNA gene in these two phyla is smaller than two copies (Větrovský & Baldrian, 2013), the strains were accepted as suitable for the study and the number of 16S rRNA gene copies estimated to be three for both.

Table 7 - Genome information from standard strains of each taxonomic group of interest – The genome size and number of 16S rRNA gene copies were used to calculate, with the DNA concentration of the standard, the number of gene copies present in the sample. This number will be applied in the preparation of the standard curve of the qPCR. The assembly level was verified to check if the genome was complete.

Species	**GenBank assembly accession**	**Assembly level**	**Genome size (bp)**	**Number of 16S rRNA gene copies**
Escherichia coli ATCC 25922	GCA_000743255.1	Complete Genome	5203440	7
Humibacter albus SC-083	GCA_000421825.1	Contigs	3693075	3
Sphingobium herbicidovorans MH	GCA_002080435.1	Complete Genome	4240798	2
Peribaccillus muralis R-8192	GCA_001645685.2	Complete Genome	5007018	9
Olivibacter domesticus DC-186	GCA_900109575.1	Scaffold	6780495	3
Bordetella petrii DSM 12804	GCA_000067205.1	Complete Genome	5287950	3

Optimization process

The optimization process to obtained optimized qPCR reactions to quantify the target taxonomic groups had as first step the performance of PCR reactions to test the primers specificity and observe the primers´ annealing temperatures. This was followed by the optimization of the qPCR reactions, varying different conditions until the acceptance criteria were meeted.

PCR assays

The primers for *Gammaproteobacteria, Betaproteobacteria, Actinomycetota*, *Bacillota and Bacteroidota* were tested using the DNA extracts from the corresponding reference strains as positive control. PCRs were performed in a TProfessional Basic Gradient thermocycler (Biometra, Dublin, Ireland). Each PCR reaction was prepared in a final volume of 20 µL and the protocols were defined according to Bacchetti De Gregoris et al., (2011). Table 5 shows the conditions and of each PCR reaction.

Table 8 - PCR optimization assays - conditions and information of the PCR reaction – The protocol 1 was applied to all the taxonomic groups in study, being three different annealing temperatures (AT) tested.

Reagents (STOCK)	**Final Vol. (uL)**	**PCR Protocol 1**	
H2O (up)	10	94ºC 5 min	
dNTP's (1mM)	4	94ºC 15 sec	x30 cycles
Taq buffer (10x)	2	**AT** 15 sec	
Primer (Fw) (10 uM)	1	72ºC 30 sec	
Primer (Rv) (10 uM)	1	72ºC 5 min	
Taq pol. (1U/uL)	1	12ºC ∞	
DNA	1		
Vol. Final	20		

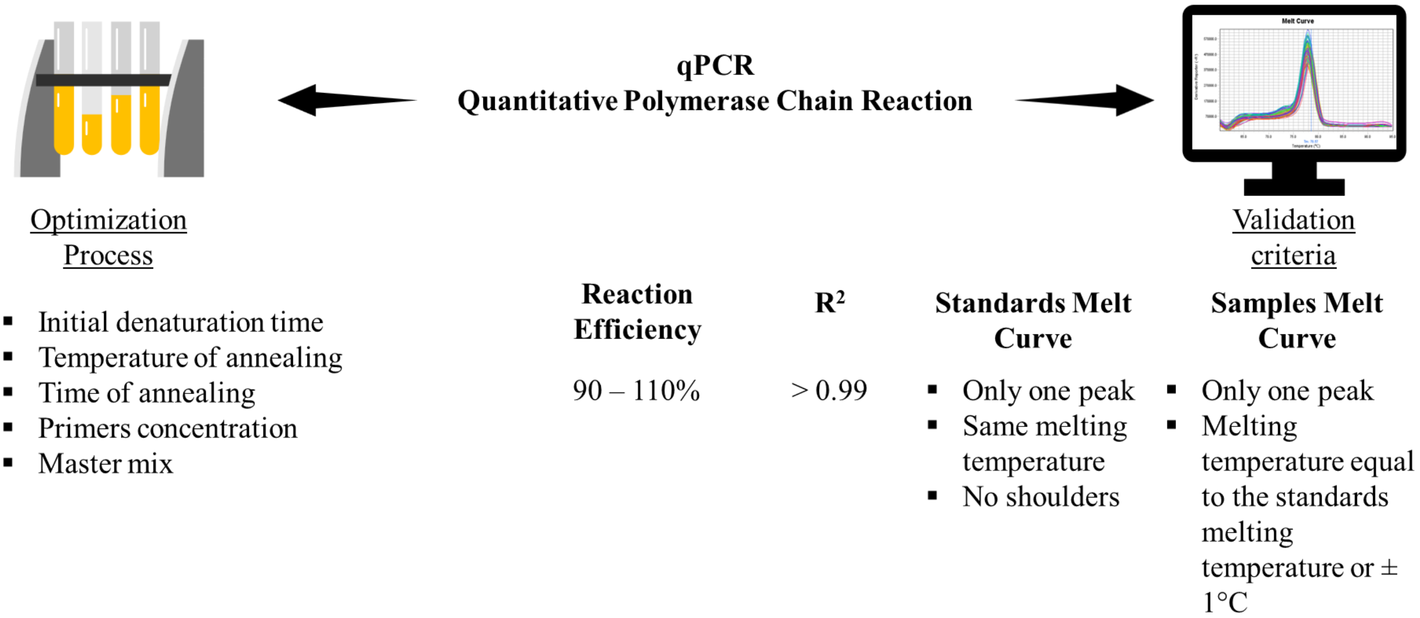
For both PCR protocols, the annealing temperatures tested were 50ºC, 55ºC and 60ºC. The PCR protocol 1 was applied to all the reactions in a first time. After the analysis of the results of the first PCR reaction, PCR protocol 1 was performed once again for the primer pairs and standard DNA of *Gammaproteobacteria* and *Actinomycetota* at an annealing temperature of 58 ºC. The PCRs were considered optimized for the set of primers when only one band was observed at the correct at the expected molecular weight.

PCR products were visualized by electrophoresis in a 1.5% agarose gel.

qPCR assay

qPCR assays quantifications were performed in all saliva samples selected for the study and in triplicate. The quantifications were conducted in a StepOnePlus™ Real-Time PCR System of 96 wells from Applied Biosystems (Life Technologies, Cartlsbad, CA, USA) applying the Standard Curve method (Brankatschk et al., 2012). The criteria defined for acceptance of the diverse quantifications were standard curve efficiency between 90% and 110%, Ct values (cycle threshold - number of cycles required for the fluorescent signal to cross the threshold being inversely proportional to quantity of target in the sample) (ThermoFisher, n.d.) from each sample possible to interpolated in the standard curve, melting curve and melting temperature identical to all the standards, melting temperature of tested samples equal to the melting curve of standard curve and absence of shoulders(Rocha & Manaia, 2020). Data analysis was performed using the StepOne

Software v2.3 supplied by Applied Biosystems and Excel. Figure 6 summarizes the qPCR optimization process and acceptance criteria considered.



qPCR
Quantitative Polymerase Chain Reaction

Optimization Process

- Initial denaturation time
- Temperature of annealing
- Time of annealing
- Primers concentration
- Master mix

Validation criteria

Reaction Efficiency

90 – 110%

R²

> 0.99

Standards Melt Curve

- Only one peak
- Same melting temperature
- No shoulders

Samples Melt Curve

- Only one peak
- Melting temperature equal to the standards melting temperature or ± 1°C

Figure 6 - Summary of the qPCR optimization process - variables tested and acceptance criteria

16S rRNA gene quantification

For the determination of total bacteria, the 16S rRNA gene was quantified by qPCR with the universal primers 1114F and 1275R and using KAPA SYBR® FAST ABI Prism® qPCR Master Mix (2X, Kapa Biosystems, Massachusetts, USA). Conditions for the 16S rRNA qPCR reactions are listed in Table 8.

Escherichia coli ATCC 25922 DNA, extracted previously in the laboratory, was used as standard DNA for the production of the standard curve. Dilutions from 10^{-1} up to 10^{-5} of the standard DNA were used by making serial dilutions of 1:10. The standard quantification was also performed in triplicate.

On a first try, original DNA extracted from saliva samples was quantified but, considering the results obtained that showed that the majority of samples´ cycle thresholds (CTs) were out of the standard curve, dilutions of 10X and 100X were prepared and quantified following the steps discussed above. The two dilutions were analysed to verify the possibility of reaction inhibition.

Taxonomic Groups Quantification using genomic DNA from each different selected

The taxonomic groups *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidota*, *Bacillota* and *Actinomycetota* were quantified by the amplification of phylum specific regions of the 16S rRNA gene. Quantifications were performed with KAPA SYBR® FAST ABI Prism® qPCR Master Mix (2X, Kapa Biosystems, Massachusetts, USA) and the specific pair of primers to each taxonomic group chosen.

The DNA extracted from reference strains was used to prepare calibration curves for each taxonomic group, using the serial dilutions observed to be adequate in the optimization processes.

Table 9 shows the different programs and conditions established for each taxonomic groups quantification in the samples.

Table 9 – 16S rRNA gene and Taxonomic Groups qPCR quantification conditions – Limit of quantification (LOQ) is lowest number of gene copies possible to determine by the qPCR reaction applied in the samples. The LOQ was calculated multiplying the reaction LOQ (ncopies/uL) per extraction volume (uL) and dividing it by the sample volume.

Taxonomic Group/Target	**qPCR Standard**	**Primers**	**|Primers| (nM)**	**Primers reference**	**qPCR reaction conditions**	**Samples Limit of Quantification (Nº of copies)**
16S rRNA	*E. coli* ATCC 25922	1114F	200	Denman & McSweeney, 2006	95ºC 10min (1x); 95ºC 15sec - 55ºC 20sec - 72ºC 10sec (35x); melting curve	1.6 x 10^4
		1275R				
Betaproteobacteria	*B. petrii* DSM 12804	Beta359f	400	Pfeiffer *et al.*, 2014	95ºC 10min (1x); 95ºC 15sec - 60ºC 20sec - 72ºC 30sec (45x); melting curve	5.5 x 10^3
		Beta682r				
Gammaproteobacteria	*E. coli* ATCC 25922	1080γF	200	De Gregoris *et al.*, 2011	95ºC 10min (1x); 95ºC 15sec - 54ºC 20sec - 72ºC 30sec (40x); melting curve	1.3 x 10^4
		γ1202R				
Actinomycetota	*H. albus* SC-083 DSM 18994	Actino235F	200	Fierer *et al.*, 2005	95ºC 10min (1x); 95ºC 15sec - 60ºC 20sec - 72ºC 30sec (40x); melting curve	1.0 x 10^5
		Eub518R				
Bacteroidota	*O. domesticus* DC-186 CCUG 54353	Cfb319	200	Fierer *et al.*, 2005	95ºC 10min (1x); 95ºC 60sec - 65ºC 30sec - 72ºC 60sec (40x); melting curve	3.3 x 10^4
		Eub518R				
Bacillota	*P. muralis* R-8192 DSM 16288	S-P-Firm-0352-a-S-18	200	Pfeiffer *et al.*, 2014	95ºC 10min (1x); 95ºC 15sec - 55ºC 20sec - 72ºC 30sec (40x); melting curve	9.6 x 10^4
		S-P-Firm-0525-a-A-18				

Statistical Analyses

The data collected from the questionnaire was organized as a binary matrix. The different categories of answers were clustered and associated to 0 or 1. “No” answers, the male gender, age below 25 years old and habit frequencies that rarely happened or happened weekly were transformed in “0” while the others were associated with the number “1”.

The statistical analyses were performed using the IBM SPSS Statistics version 28 (IBM Corp., Armonk, NY, USA). Kolmogorov-Smirnov was applied to test if the data followed a normal distribution. Since normal distribution of the data was not verified, non-parametric methods were applied. The Mann-Whitney U test was used to assess the occurrence of significant differences between the two different categories created for each lifestyle characteristic (0 and 1, with different meanings for each characteristic), with a significance level of $p<0.01$. Tables with the statistical calculations for the different analyses are present in the results section. All the statistical analysis were performed with non-logarithm values.

The bacterial group named as “Other Groups” was calculated subtracting to the mean 16S rRNA value of each sample the sum of the bacterial taxonomic groups quantified by qPCR in this study. For this reason, for the category Others it is not show standard deviation.

Redundancy analysis (RDA) was performed with Canoco 5.01 software (Smilauer & Leps, 2014). This analysis was done to assess the existence of relationships between the bacterial composition of the saliva samples, bacterial groups ratios and the possible microbiota shaping factors. Variables with $p<0.01$ were forward selected and 1000 Monte Carlo permutations were done.

Results

Background information

Saliva samples were supplied by 51 young adults, students of dental medicine at University of Porto, Portugal. The characteristics and habits enquired to participants are listed in Table 10.

Table 10 - Characteristics and habits reported by 51 study participants in a online query that followed the declaration of informed consent for study participation.

Personal Characteristics	**Age**	**Years**	**Sex**	**% (N)**
	Median	20	Male (0)	19.6 % (10)
	Range	18 - 33	Female (1)	80.4% (41)
Health Conditions	**Antibiotic Intake in the last 12 months**	**N**	**CPO**	**N**
	No (0)	35	0 or 1 (0)	21
	Yes (1)	16	≥ 2 (1)	30
Hygiene Habits	**Daily toothbrushing**	**N**	**Tongue brushing**	**N**
	Up to 2x/day (0)	41	Never to weekly (0)	24
	Between 3x up >4x/day (1)	10	Daily (1)	27
	Gum brushing	**N**	**Use of Eletric Toothbrushing**	**N**
	Never to weekly (0)	36	No (0)	36
	Daily (1)	15	Yes (1)	10
	Use of Dental floss	**N**		
	Never to weekly (0)	35		
	Daily floss (1)	16		
Diet Habits	**Coffee consumption**	**N**	**Milk consumption**	**N**
	Never to 1/2x per week	21	Never to 1/2x a week (0)	20
	3/5x per week up to 2x/day	30	3/5x a week to >2x/day (1)	31
	Soft drinks intake	**N**	**Meat consumption**	**N**
	Never to weekly (0)	32	Never to monthly (0)	3
	more than 1x/week	19	1x/week to 1/2x/day (1)	48
	Fish consumption	**N**	**Chewing gum consumption**	**N**
	Never to monthly (0)	11	Never to weekly (0)	40
	1x/week to 1/2x/day (1)	40	1x/week to >1x/day (1)	11
Others	**Onychophagia**	**N**	**Smoking habits**	**N**
	No/Occasionally (0)	42	Never to weekly (0)	49
	Yes (1)	9	At least 1 cigarette/day (1)	2
	Pen bitting	**N**	**Use of eletric cigarette**	**N**
	No (0)	27	No (0)	49
	Yes/Occasionally (1)	24	Yes (1)	2

The questionnaire included 68 questions, of which 19 were considered relevant for discussion of the results (Table 10). The other 49 questions addressed topics such as face length, intake of medication for anxiety, sleeping hours, breastfeed age, type of birth, menstrual phase at the time of sampling. These were not considered because accordingly to the analysed literature have not demonstrated yet to have direct impact in the oral cavity microbiota therefore were not considered relevant for this study and some topics did not have representative different answers in the subject group.

Query answers were converted into a binary (positive = 1 /negative = 0) table (Table 10). Based on a univariate analysis with the Mann-Whitney U test the comparison of negative and positive answers evidenced significant differences between both groups for sex, antibiotic intake, daily toothbrushing, use of electric toothbrush, dental floss, onychophagia, pen biting, use of electronic cigarette, coffee and soft drinks intake, fish consumption and chewing gum consumption. The features age, carious, lost and clogged teeth index, tongue brushing, gum brushing and meat consumption did not evidence significant differences in terms of taxonomic groups ratio.

Samples' characterization

DNA concentrations in the analysed extracts ranged from 4.69 ng/µL to 285 ng/ µL, with quite variable values among samples (Figure 7). The abundance of the 16S rRNA gene, used to assess total bacterial quantity, varied between 7.3 and 10.0 log-units (log of gene copy number /mL sample) (Figure 10A). These values were independent of the DNA concentration in the extract (Figure 8).

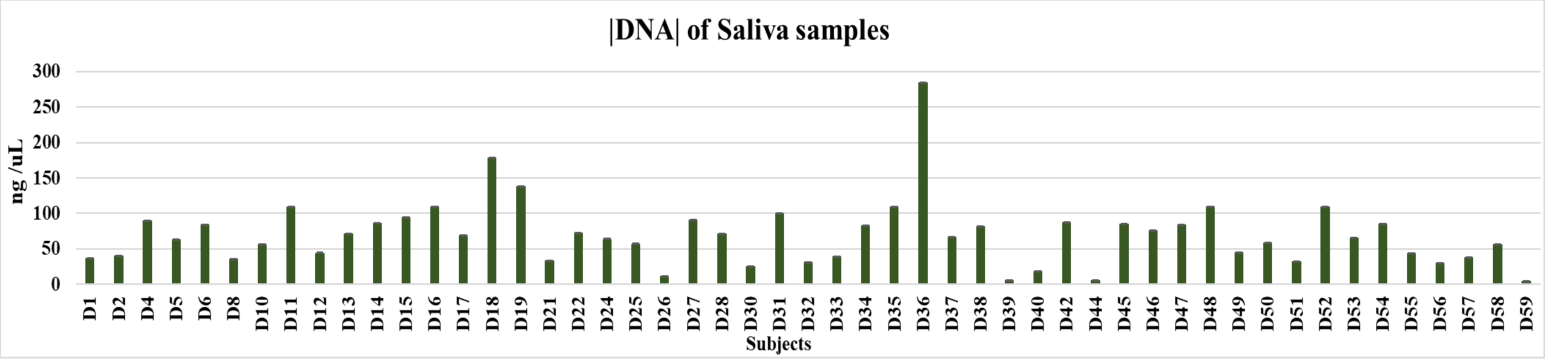


Figure 7 - DNA concentration of the different saliva samples

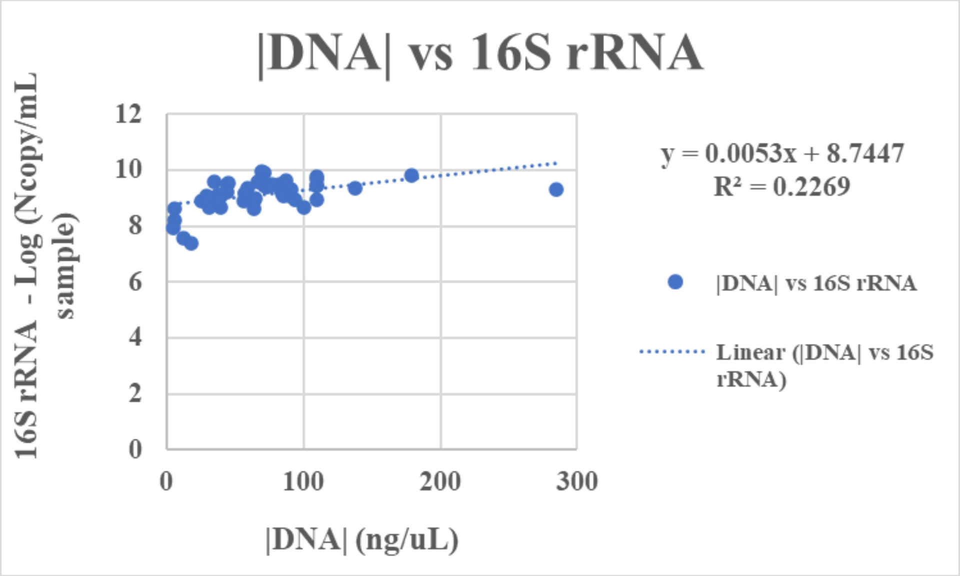


Figure 8 - Relation of the DNA concentration versus the 16S rRNA quantification.

Aiming at a better understanding of the bacterial groups prevailing in oral cavity and how these can be influenced by external factors (host characteristics or habits), the quantification of the 16S rRNA gene targeting total bacteria was specified for members of the phyla *Actinomycetota, Bacillota,* and *Bacteroidota* and the *Pseudomonadota* classes *Gammaproteobacteria, Betaproteobacteria*. In all individuals, phyla/classes abundance mean values (log-units /mL saliva) could be ranked as *Bacteroidota* > *Actinomycetota* > *Gammaproteobacteria* > *Betaproteobacteria* > *Bacillota* (Figure 10).

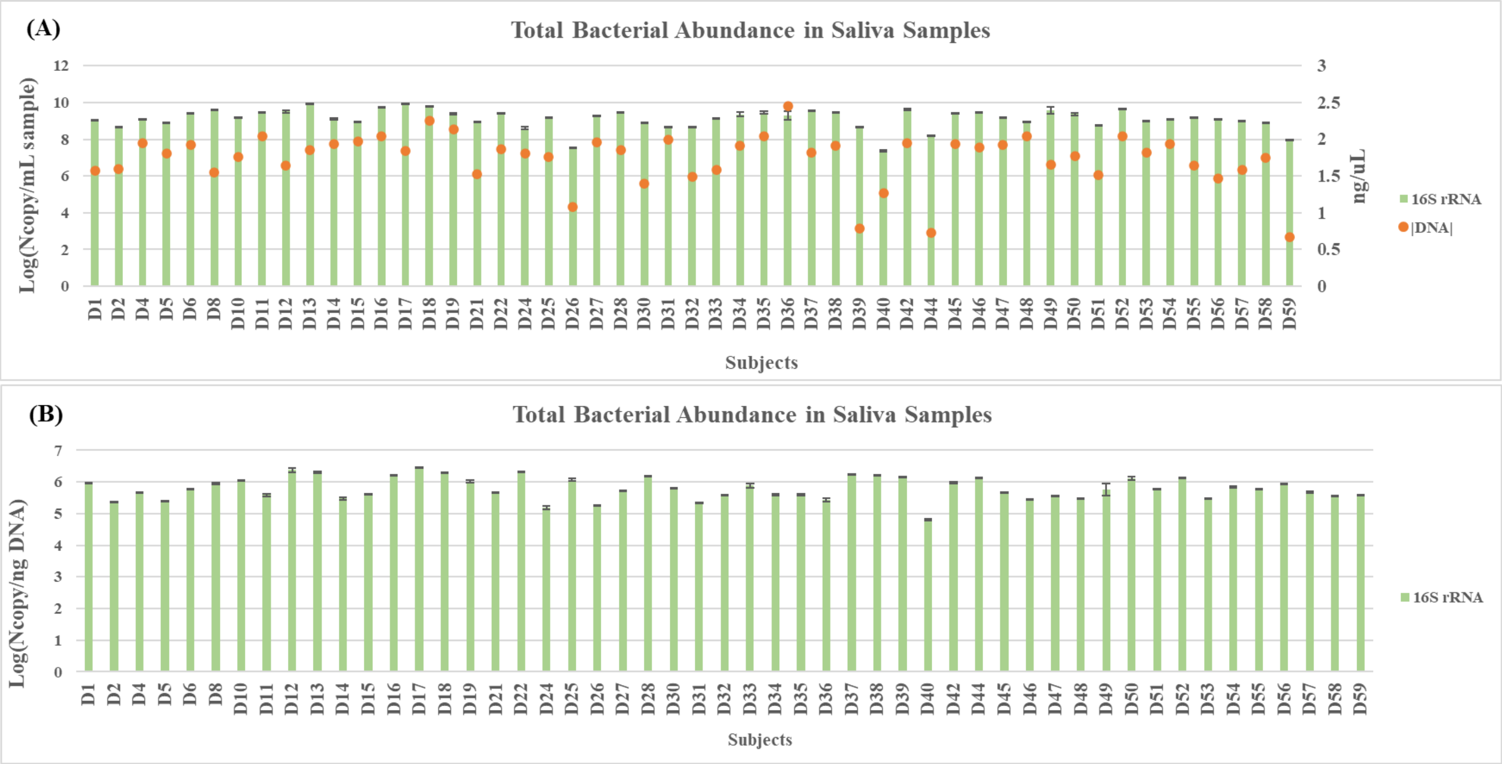


Figure 9 - Abundance of total bacteria measured in saliva samples based on 16S rRNA gene copy number, expressed as log-units gene copy number per mL saliva (A) or per ng DNA (B) and DNA concentration in the saliva extracts (ng/µL).

This ranking was also observed when the results were expressed in log-units of gene copy number per taxa / 16S rRNA gene copy number (Figure 11). Considering the average values for all individuals, *Bacteroidota* represented 8.2 ± 0.6 log-units (ncopy/mL), *Actinomycetota* 7.9 ± 0.6 log-units (ncopy/mL), *Gammaproteobacteria* 7.5 ± 0.6 log-units (ncopy/mL), *Betaproteobacteria* 7.3± 0.7 log-units (ncopy/mL) and *Bacillota* 7.1 ± 0.6 log-units (ncopy/mL). The designation “Others Groups” corresponds to the difference between the 16S rRNA gene quantification and the sum of the five phyla in each individual. The mean value for the “Other” was 9.0 log-units (ncopy/mL), having a mean higher than any other group. *Betaproteobacteria* was the phylum for which most variation was observed among individuals, with 3.4 log-units (ncopy/mL) of difference between the lowest and highest value. The opposite was observed for the phylum *Bacillota* with 2.6 log-units (ncopy/mL) of variation.

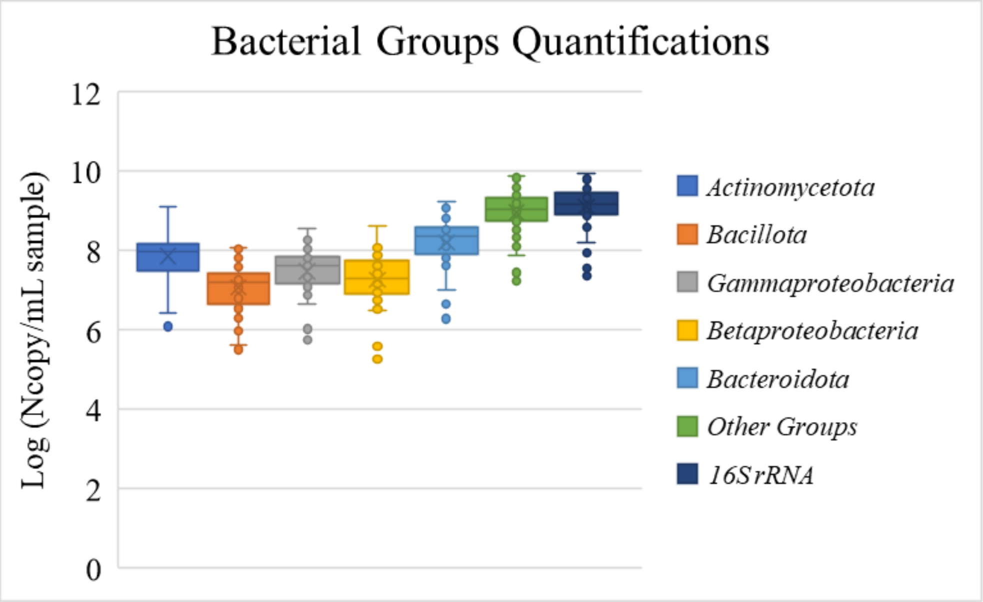


Figure 10 - Bacterial groups in study mean quantifications overview in ncopy per mL of sample.

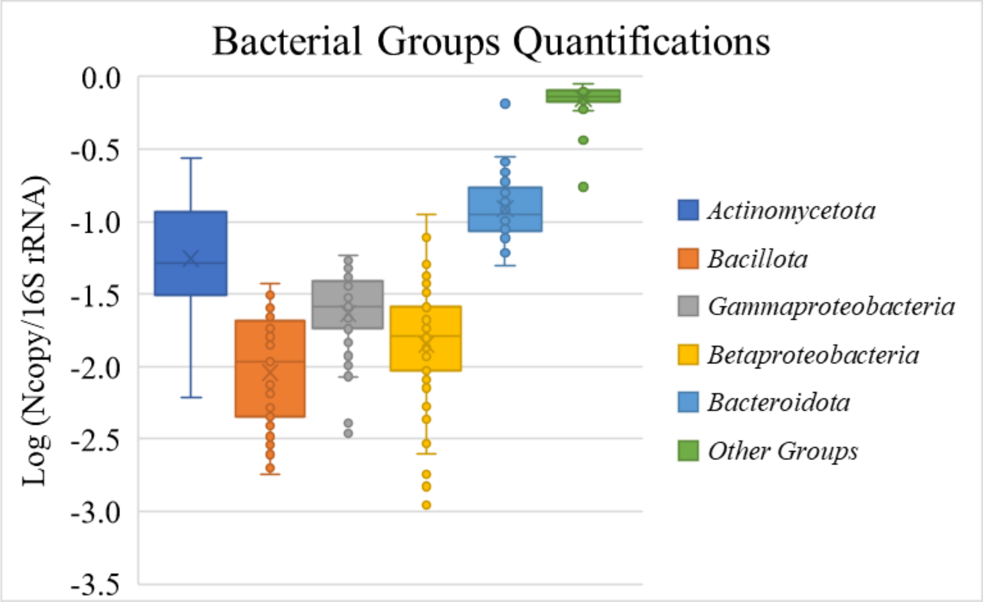


Figure 11 – Bacterial groups in study prevalence overview in ncopy per 16S rRNA.

The quantification of *Bacteroidota,* Figure 12A, ranged from 6.3 log-units, in the sample D40, to 9.3 log-units of the sample D18 as demonstrated in figure 8. The 5 highest values of quantification were verified in sample D18 (9.3 log-units), D52 (9.1 log-units), D17 (9.0 log-units), D4 (8.9 log-units) and D13 (8.9 log-units).

Figure 12B shows the quantification of *Actinomycetota*. For this phylum the sample with the lowest quantification was D26 (6.1 log-units) and the sample with the highest value was D52 with 9.1 log-units. The 5 samples with highest quantifications had *Actinomycetota* values higher than 8.49 log-units (D38, D35, D49, D46 and D52 in crescent order).

Gammaproteobacteria was the third bacterial group with the highest mean quantification values. Sample D18 had the maximum value for this group (8.6 log-units), while sample D44 had the lowest (5.8 log-units). The 5 samples with highest values were D35 (8.1 log -units), D46 (8.1 log-units), D13 (8.3 log-units), D52 (8.4 log-units) and D18, the sample with the maximum value.

The values of *Betaproteobacteria* quantification ranged from 5.3 log-units (sample D44) up to 8.6 log-units (sample D37) as demonstrated in figure 12D. The samples with higher quantification for this taxonomic bacterial group presented values superior to 8.1 log-units (D13 – 8.1 log-units, D50 – 8.1 log-units, D49 – 8.2 log-units, D17 - 8.3 log-units and D35).

Figure 12E represents the different quantifications obtained for *Bacillota* phylum, the one with the lowest mean value. The interval for values of quantification was 5.5 log-units (D40) to 8.1 log-units (D52). In crescent order of values, the following samples presented the highest quantifications: D38 (7.8 log-units), D34 (7.9 log-units), D35 (7.9 log-units), D42 (8.1 log-units) and D52.

The "others" bacterial groups was calculated by subtracting these quantified groups of the 16S rRNA. Higher sum of the different groups' quantifications leads to lower other groups values. The quantifications ranged from 7.23 log-units, sample D40, to 9.88 log-units, sample D17. The samples with highest values were D42 with 9.51 log-units, D18 with 9.61 log-units, D16 with 9.69 log-units, D13 with 9.84 log-units and D17.

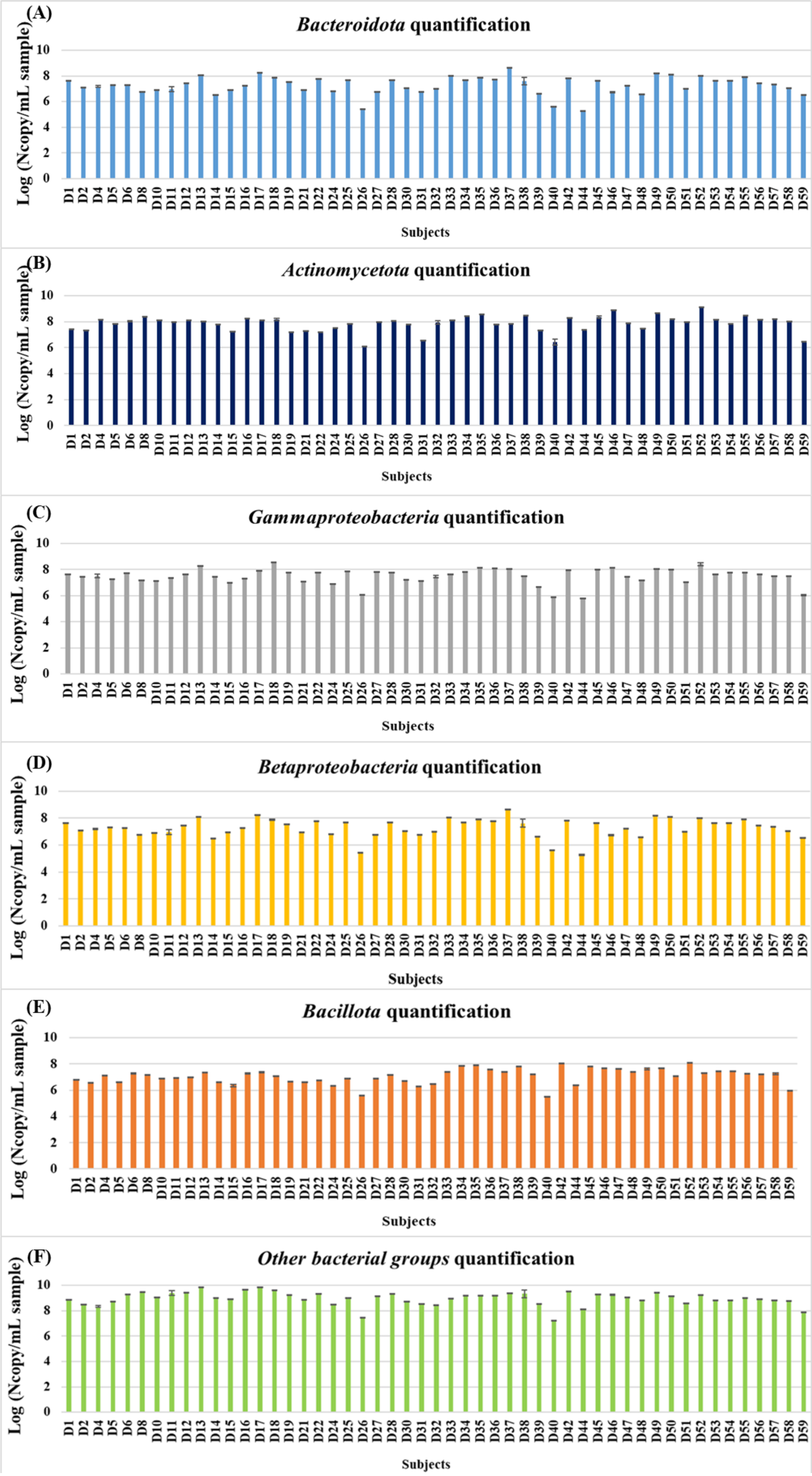


Figure 12 - Taxonomic groups qPCR quantification in log-units (ncopy/mL sample) – (A) Bacteroidota Abundance; (B) Actinomycetota Abundance; (C)Gammaproteobacteria Abundance; (D) Betaproteobacteria Abundance; (E) Bacillota Abundance; (F) Other bacterial groups Abundance.

To better understand the results obtained, the data was normalized by the 16S rRNA quantifications. The ratios obtained by that normalization are represented in figure 13 and Table 11.

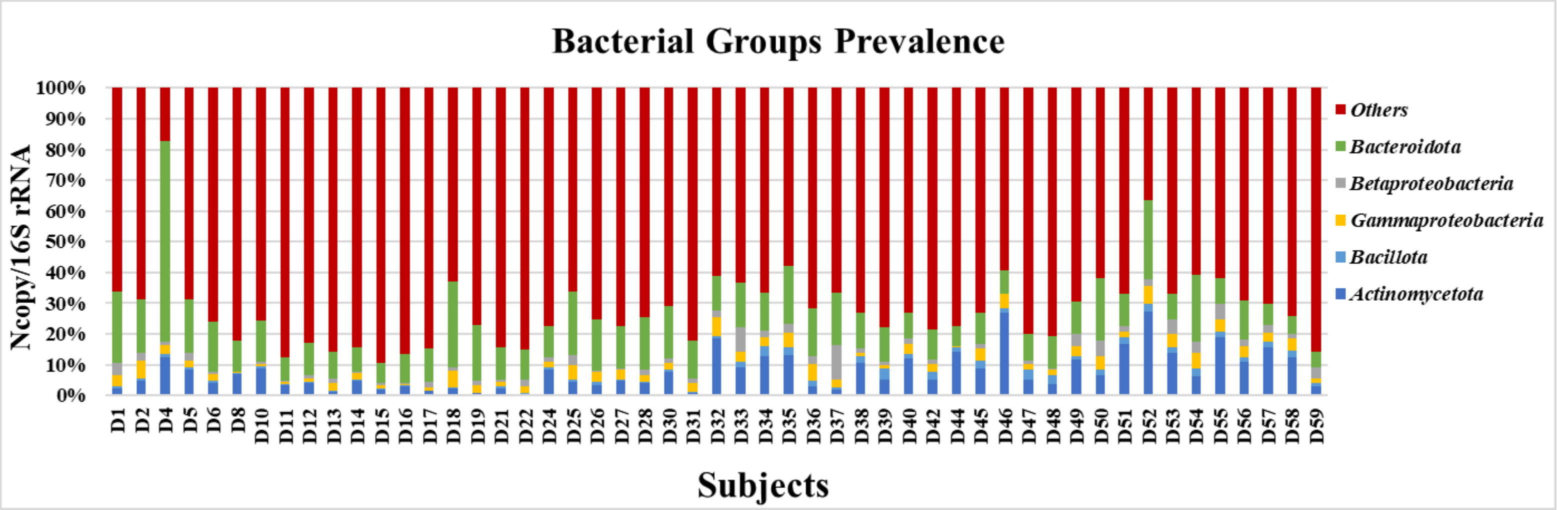


Figure 13 – Bacterial groups prevalence obtain by the data normalization per 16S rRNA.

The percentage of others bacterial groups in relation to total bacteria presented a mean value of 0.7. Samples D4 and D52 had the lowest ratio values for “other” bacterial groups ratio, 0.2 and 0.4, respectively and D15 (0.9) and D11 (0.9) were the samples with the highest ratios.

Bacteroidota presented a ratio mean value of 0.1, with the rest of the samples with values between 0.1 and 0.7. This phylum had the largest range of values. Saliva samples of subjects D4, D18, D52 and D54 had the highest ratios of the *Bacteroidota* phylum (0.7, 0.3, 0.3 and 0.5, respectively). The samples D59, with a value of 0.1, and D58/D44/D15 with a ratio of 0.1 are the lowest values.

With an interval of values between 0.01-0.3, the *Actinomycetota* phylum has a mean value of 0.08. Ratio values of 0.19 (sample D32 and D55) and 0.3 (sample D46 e D52) were the highest verified for this phylum while samples D19, D22 and D31 had the lowest ratios (<0.01).

The bacterial groups with the lowest mean values were *Gammaproteobacteria*, *Betaproteobacteria* and *Bacillota*, in this order as demonstrated in figure 14.

With a mean value of 0.03, the values of the ratios in the *Gammaproteobacteria* bacterial group ranged from 0.003 up to 0.06. Samples D2, D18, D32 and D52 presented the highest ratio, 0.06. Samples D8, D16 and D44 had ratios lower than 0.01, being the lowest.

Table 11 – Prevalence of the different target taxonomic groups – Prevalence was calculated dividing the abundance of the taxonomic group (ncopy /mL of sample) per the 16S rRNA; Standard deviation values ranges were the following: Actinomycetota : 0.00 – 0.05; Bacillota: 0.00; Gammaproteobacteria: 0.00 – 0.02; Betaproteobacteria: 0.00 – 0.01 and Bacteroidota: 0.00-0.06.

Samples	**Prevalence of the target bacterial taxonomic groups (ncopy / 16S rRNA)**					
	Actinomycetota	***Bacillota***	***Gammaproteobacteria***	***Betaproteobacteria***	***Bacteroidota***	**Other bacterial groups**
D1	0.02	0.01	0.04	0.04	0.23	0.66
D2	0.05	0.01	0.06	0.03	0.17	0.69
D4	0.12	0.01	0.03	0.01	0.65	0.17
D5	0.09	0.01	0.02	0.03	0.17	0.69
D6	0.04	0.01	0.02	0.01	0.16	0.76
D8	0.07	0.00	0.00	0.00	0.10	0.82
D10	0.09	0.01	0.01	0.01	0.13	0.76
D11	0.03	0.00	0.01	0.00	0.08	0.87
D12	0.04	0.00	0.01	0.01	0.11	0.83
D13	0.01	0.00	0.02	0.01	0.09	0.86
D14	0.05	0.00	0.02	0.00	0.08	0.84
D15	0.02	0.00	0.01	0.01	0.06	0.89
D16	0.03	0.00	0.00	0.00	0.10	0.86
D17	0.01	0.00	0.01	0.02	0.11	0.85
D18	0.02	0.00	0.06	0.01	0.28	0.63
D19	0.01	0.00	0.03	0.01	0.18	0.77
D21	0.02	0.00	0.01	0.01	0.10	0.84
D22	0.01	0.00	0.02	0.02	0.10	0.85
D24	0.08	0.01	0.02	0.02	0.10	0.77
D25	0.05	0.01	0.05	0.03	0.20	0.66

Samples	**Prevalence of the target bacterial taxonomic groups (ncopy / 16S rRNA)**					
	Actinomycetota	***Bacillota***	***Gammaproteobacteria***	***Betaproteobacteria***	***Bacteroidota***	**Other Bacterial Groups**
D26	0.03	0.01	0.03	0.01	0.17	0.75
D27	0.05	0.00	0.03	0.00	0.14	0.78
D28	0.04	0.01	0.02	0.02	0.17	0.74
D30	0.08	0.01	0.02	0.01	0.17	0.71
D31	0.01	0.00	0.03	0.01	0.12	0.82
D32	0.19	0.01	0.06	0.02	0.11	0.61
D33	0.09	0.02	0.03	0.08	0.15	0.63
D34	0.13	0.03	0.03	0.02	0.12	0.67
D35	0.13	0.03	0.05	0.03	0.19	0.58
D36	0.03	0.02	0.05	0.03	0.16	0.71
D37	0.02	0.01	0.03	0.11	0.17	0.66
D38	0.11	0.02	0.01	0.02	0.12	0.73
D39	0.05	0.04	0.01	0.01	0.11	0.78
D40	0.12	0.01	0.03	0.02	0.08	0.73
D42	0.05	0.03	0.02	0.02	0.10	0.79
D44	0.14	0.01	0.00	0.00	0.06	0.78
D45	0.09	0.03	0.04	0.02	0.10	0.73
D46	0.27	0.02	0.04	0.00	0.08	0.59
D47	0.05	0.03	0.02	0.01	0.09	0.80
D48	0.04	0.03	0.02	0.00	0.11	0.81
D49	0.12	0.01	0.03	0.04	0.10	0.70
D50	0.07	0.02	0.04	0.05	0.20	0.62
D51	0.17	0.02	0.02	0.02	0.11	0.67
D52	0.27	0.03	0.06	0.02	0.26	0.36

Samples	**Prevalence of the target bacterial taxonomic groups (ncopy / 16S rRNA)**					
	Actinomycetota	***Bacillota***	***Gammaproteobacteria***	***Betaproteobacteria***	***Bacteroidota***	**Other Bacterial Groups**
D53	0.14	0.02	0.04	0.04	0.09	0.67
D54	0.06	0.02	0.05	0.04	0.22	0.61
D55	0.19	0.02	0.04	0.05	0.08	0.62
D56	0.11	0.02	0.04	0.02	0.13	0.69
D57	0.16	0.02	0.03	0.02	0.07	0.70
D58	0.12	0.02	0.04	0.01	0.06	0.74
D59	0.03	0.01	0.01	0.04	0.05	0.86

The *Betaproteobacteria* group values for the ratios, ncopy per 16S rRNA, ranged from 0.001 to 0.1, with a mean value of 0.02. The lowest ratio was lower than 0.01 and it was verified in samples D8, D11, D14, D16, D27, D44, D46 and D48 while samples D37 and D33 presented the highest values (0.11 and 0.08, respectively).

Finally, *Bacillota* showed the lowest mean value for the ratio with 16S rRNA, 0.01. The ratios in the samples were in the values interval of 0.0018 to 0.04. Samples D8, D11, D12, D13, D14, D15, D16, D17, D18, D19, D21, D22 and D31 had ratio value lower than 0.01. Sample D39 were the samples with the highest ratio, 0.04. Samples D34, D35, D45, D47, D48 and D52 were the sample with the next highest ratio (0.03).

Inference of characteristics or habits associated with specific bacterial groups

Data for the different bacterial groups was organized and analysed statistically. The data did not follow a normal distribution as observed in Table 12. Therefore, further analyses were done using non-parametric tests.

Table 12 – Results obtained from the analysis performed to evaluate the normal distribution of the quantification results – significance level of $p<0.01$.

Tests of Normality

	Kolmogorov-Smirnov[a]			**Shapiro-Wilk**		
	Statistic	df	Sig.	Statistic	df	Sig.
16S rRNA (ncopy/ml)	0.169	153	0.000	0.836	153	0.000
***Actinomycetota* (ncopy/mL)**	0.262	153	0.000	0.582	153	0.000
***Bacillota* (ncopy/mL)**	0.221	153	0.000	0.759	153	0.000
***Gammaproteobacteria* (ncopy/mL)**	0.192	153	0.000	0.720	153	0.000
***Betaproteobacteria* (ncopy/mL)**	0.251	153	0.000	0.605	153	0.000
***Bacteroidota* (ncopy/mL)**	0.181	153	0.000	0.755	153	0.000
Other Bacterial Groups (ncopy/mL)	0.160	153	0.000	0.777	153	0.000
Actinomycetota/16S rRNA	0.170	153	0.000	0.871	153	0.000
Bacillota/16S rRNA	0.187	153	0.000	0.891	153	0.000
Gammaproteobacteria/16S rRNA	0.114	153	0.000	0.967	153	0.001
Betaproteobacteria/16S rRNA	0.182	153	0.000	0.773	153	0.000
Bacteroidota/16S rRNA	0.167	153	0.000	0.655	153	0.000
Other bacterial groups/16S rRNA	0.129	153	0.000	0.838	153	0.000

a. Lilliefors Significance Correction

To test the hypothesis that some characteristics or habits may be associated with variations on the abundance of some bacterial groups, the binary categories defined above (section Statistical Analysis), were the basis to assess statistically significant differences of qPCR determinations for each group. The non-parametric Mann-Whitney U test indicated the existence of statistically significant differences between the two binary categories for 14 out of 19 tested features. The results of this analysis are summarized in Table 13.

The personal characteristics of the individuals, age and sex, demonstrated significative differences between the two groups for almost every taxonomic group. Two different groups of age were assessed, "0" representing age younger than 25 years old, and "1" representing ages of 25 years old and older. Significantly differences were only observed when comparing the results of the two age groups for the total bacteria abundance. Total bacterial abundance was higher in individuals with ages between 18 and 24 (2.3 x 10^9 ncopy / mL of sample) while individuals with age of 25 years old or older had lower total bacterial abundance (7.9 x 10^8 ncopy / mL of sample).

In the sex characteristic the male group demonstrated to have significantly higher quantity of total bacteria, 3.7 × 10^9 ncopies of the 16S rRNA gene per milliliter of sample while the female groups only had 1.8 × 10^9 ncopies,

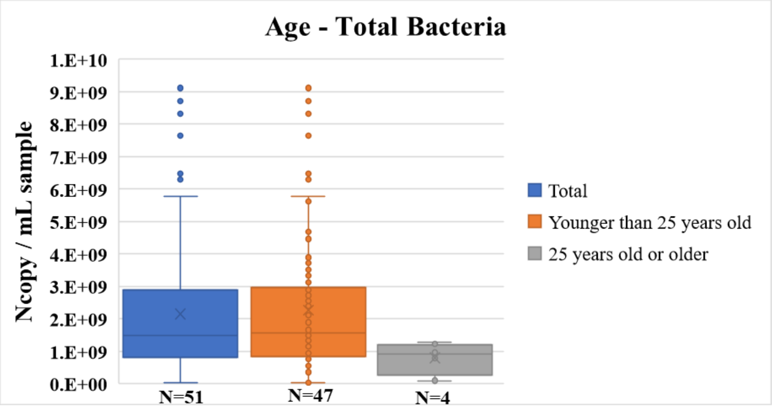


Figure 14 - Statistically different results: Age – Box-plot show the different distribution of results obtained for the total bacterial abundance significantly different (p<0.01) for the different two group ages.

Concerning the health characteristics in study, differences between the group of individuals that had took antibiotic and the group of individuals that did not take antibiotic were observed. Actinomycetota and Bacillota phyla were more frequent in people that took antibiotics in the 12 months previously to the sample collection (0.1 ncopy/16S rRNA and 0.02 ncopy/16S rRNA, respectively) than people that did not took antibiotics (0.07 ncopy/16S rRNA and 0.01 ncopy/16S rRNA, respectively).

Table 13 - Summary of the statistical analysis that compared the prevalence of each bacterial group expressed as (ncopy/16S rRNA) and total bacteria abundance (ncopy/mL sample) between the binary categories and indication of that presenting the highest value (0 or 1) as well as the corresponding number of individuals (n/51) – n.s - no significant differences observed

Subject´s Characteristics/Habits	**Total Bacteria**	***Actinomycetota***	***Bacillota***	***Gammaproteobacteria***	***Betaproteobacteria***	***Bacteroidota***	**Others**
Age	0 (47/51) p=0.008	n.s	n.s	n.s	n.s	n.s	n.s
Sex	0 (10/51) p=0.000	1 (41/51) p=0.001	1 (41/51) p=0.004	1 (41/51) p=0.000	1 (41/51) p=0.000	n.s	0 (10/51) p=0.000
Antibiotic Intake	n.s	1 (16/51) p=0.000	1 (16/51) p=0.000	n.s	n.s	n.s	n.s
CPO	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Daily toothbrushing	n.s	n.s	n.s	n.s	n.s	n.s	0 (41/51) p=0.008
Tongue brushing	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Gum brushing	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Use of eletric toothbrush	n.s	n.s	n.s	n.s	0 (36/51) p=0.007	1 (10/51) p=0.003	n.s
Use of dental floss	n.s	n.s	n.s	n.s	n.s	n.s	0 (35/51) p=0.006
Onychophagia	1 (9/51) p=0.001	0 (42/51) p=0.000	0 (42/51) p=0.000	0 (42/51) p=0.000	0 (42/51) p=0.005	n.s	1 (9/51) p=0.000
Smoking habits	n.s	n.s	n.s	n.s	n.s	n.s	n.s

Subject´s Characteristics/Habits	**Total Bacteria**	***Actinomycetota***	***Bacillota***	***Gammaproteobacteria***	***Betaproteobacteria***	***Bacteroidota***	**Others**
Pen biting	n.s	n.s	n.s	0 (27/51) p=0.000	n.s	n.s	1 (24/51) p=0.001
Smoking habits	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Eletronic cigarette	1 (2/51) p=0.007	n.s	n.s	1 (2/59) p=0.002	n.s	n.s	n.s
Coffee consumption	n.s	n.s	n.s	1 (30/51) p=0.009	n.s	n.s	n.s
Soft drinks consumption	n.s	n.s	n.s	n.s	0 (32/51) p=0.004	n.s	n.s
Milk consumption	0 (20/51) p=0.009	n.s	n.s	n.s	n.s	1 (31/51) p=0.000	n.s
Meat consumption	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Fish consumption	n.s	n.s	n.s	1 (40/51) p=0.000	1 (40/51) p=0.004	n.s	n.s
Chewing gum consumption	n.s	n.s	n.s	n.s	0 (40/51) p=0.000	n.s	n.s

antibiotic 12 months previously the beginning of the study had mean ratios of ncopy per 16S rRNA of 0.1 and 0.02, for *Actinomycetota* and *Bacillota* phyla, respectively.

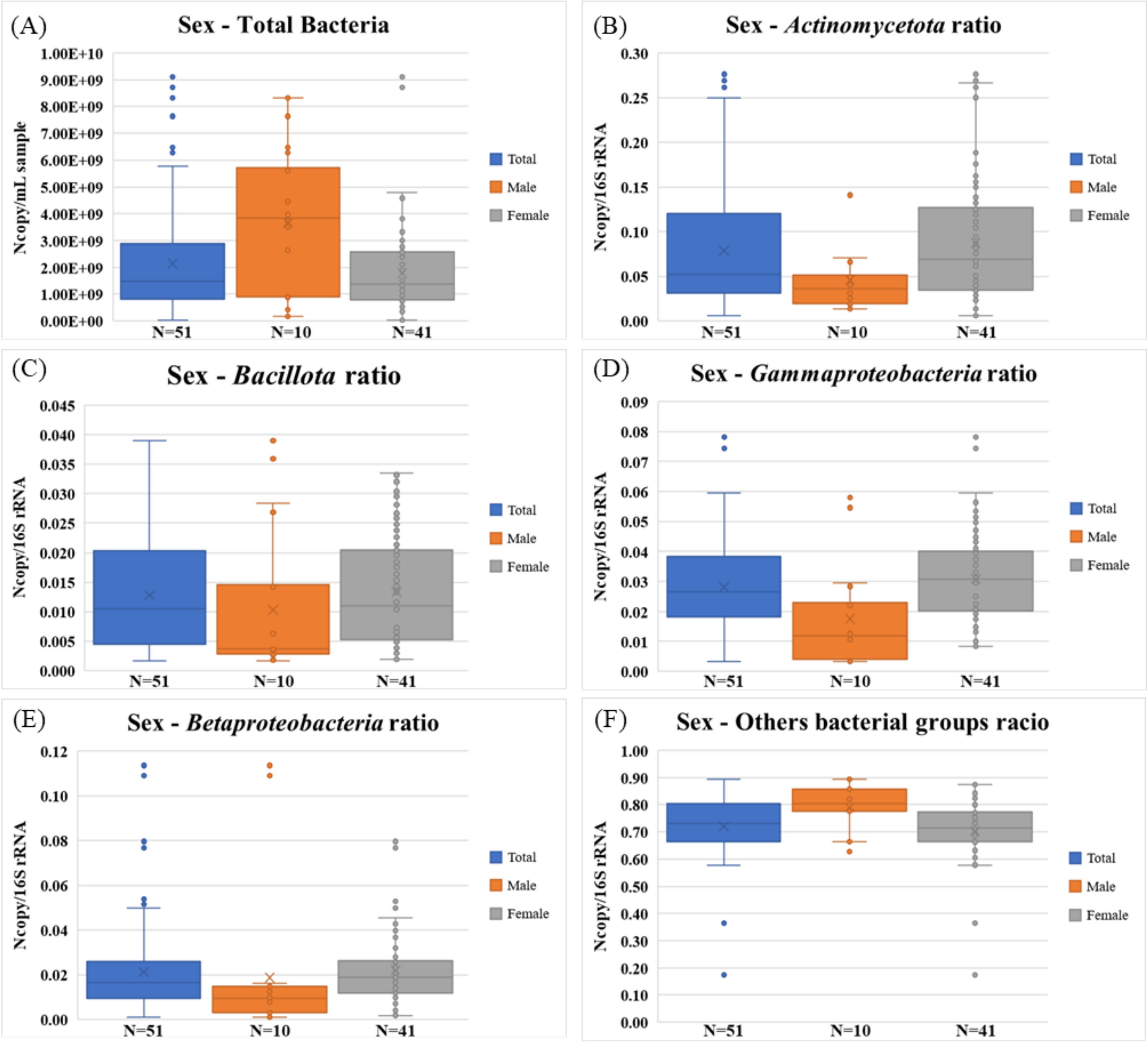


Figure 15 -Statistically different results: Sex – Box-plots show the different distribution of results obtained male and female that demonstrated significative differences (p<0.01)– (A) Total bacteria abundance; (B) Actinomycetota prevalence; (C) Bacillota prevalence; (D) Gammaproteobacteria prevalence; (E) Betaproteobacteria prevalence; (F) Other bacterial groups prevalence. Total bacteria abundance was measured by the quantification of the 16S rRNA gene. The prevalence of bacterial groups was obtained normalizing the data (ncopy/ mL of sample) by the 16S rRNA quantification.

In terms of dental hygiene, differences between the two groups (individuals that brushed up to 2 times per day versus individuals that brushed between 2 and 4 times per day; individuals that never floss or flossed weekly versus individuals that floss daily) were detected in the results concerning daily toothbrushing and dental floss. For these, higher ratios of ncopy per 16S rRNA were observed for the groups that fewer times performed the mentioned habits. The use of electric toothbrush, demonstrated significative

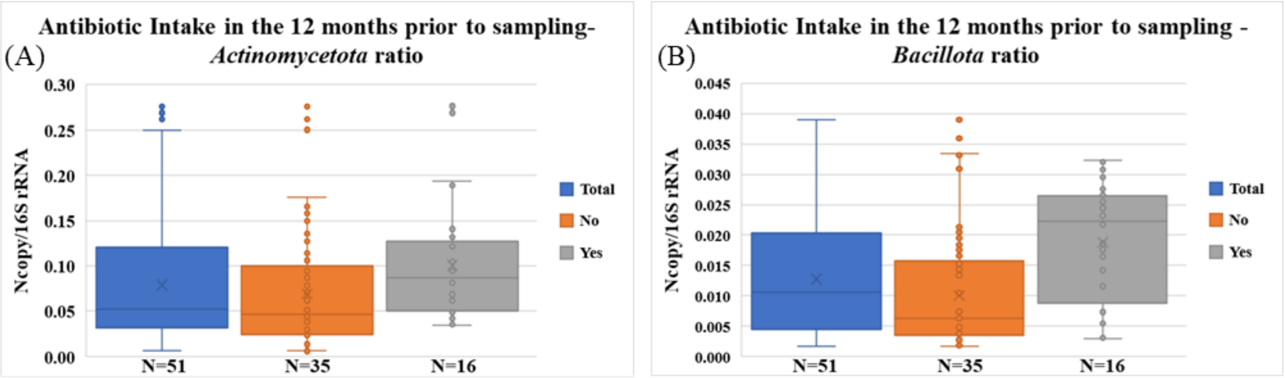


Figure 16 - Statistically different results: Antibiotic Intake - Box-plots show the different distribution of results obtained for the bacterial groups that demonstrated significative differences (p<0.01) between subjects that took antibiotics in the last 12 months prior to the beginning of this study and subjects that had no antibiotic intake. (A) Actinomycetota prevalence; (B) Bacillota prevalence. The prevalence of bacterial groups was obtained normalizing the data (ncopy/ mL of sample) by the 16S rRNA quantification.

differences between the group of individuals that used it for the phyla *Betaproteobacteria* and *Bacteroidota*. *Betaproteobacteria* was more frequent in individuals that did not use an electric toothbrush (0.02 ncopy/16S rRNA) while the *Bacteroidota* phylum was more frequent in individuals that used an electric toothbrush (0.20 ncopy/16S rRNA). Five individuals did not respond to this topic.

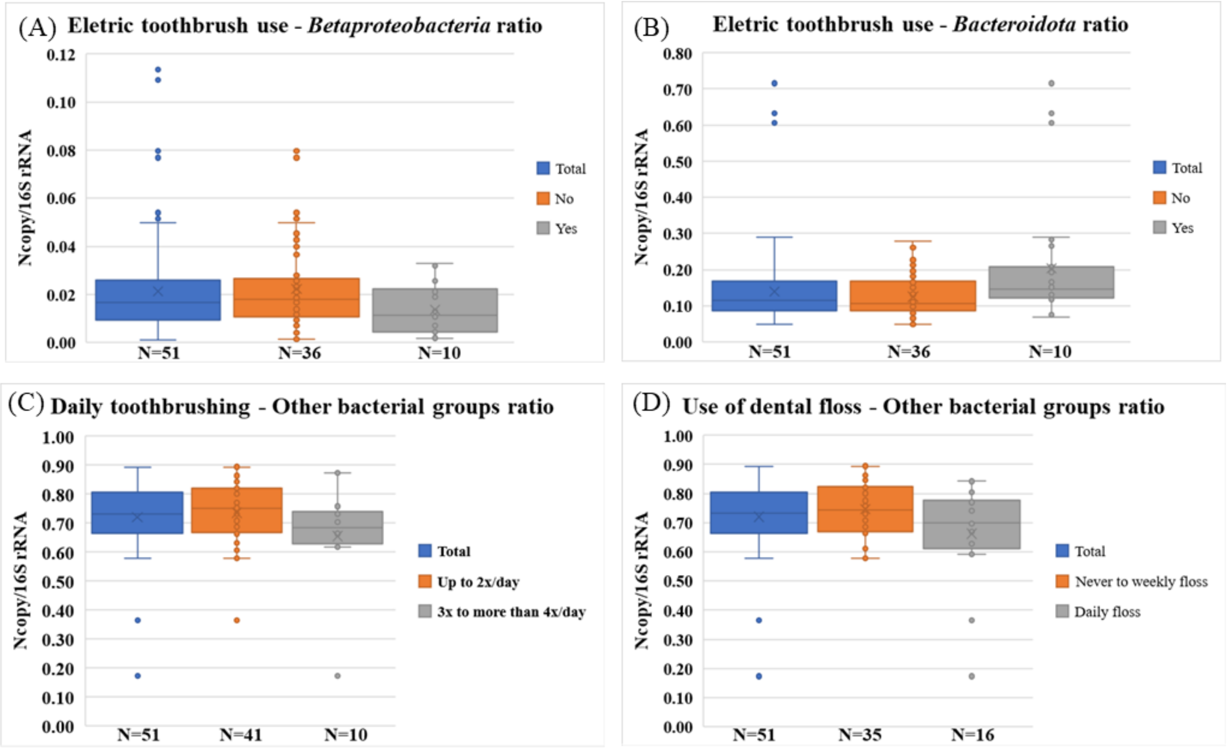


Figure 17 - Statistically different results for the hygiene habits: Box-plots show the different distribution of results obtained for the bacterial groups that demonstrated significative differences (p<0.01) between groups of subjects (A) Betaproteobacteria prevalence in Eletric toothbrush use; (B) Bacteroidota prevalence in Eletric toothbrush use; (C) Others bacterial groups prevalence in Daily toothbrushing; (D) Other bacterial groups prevalence in use of dental floss. The prevalence of bacterial groups was obtained normalizing the data (ncopy/ mL of sample) by the 16S rRNA quantification.

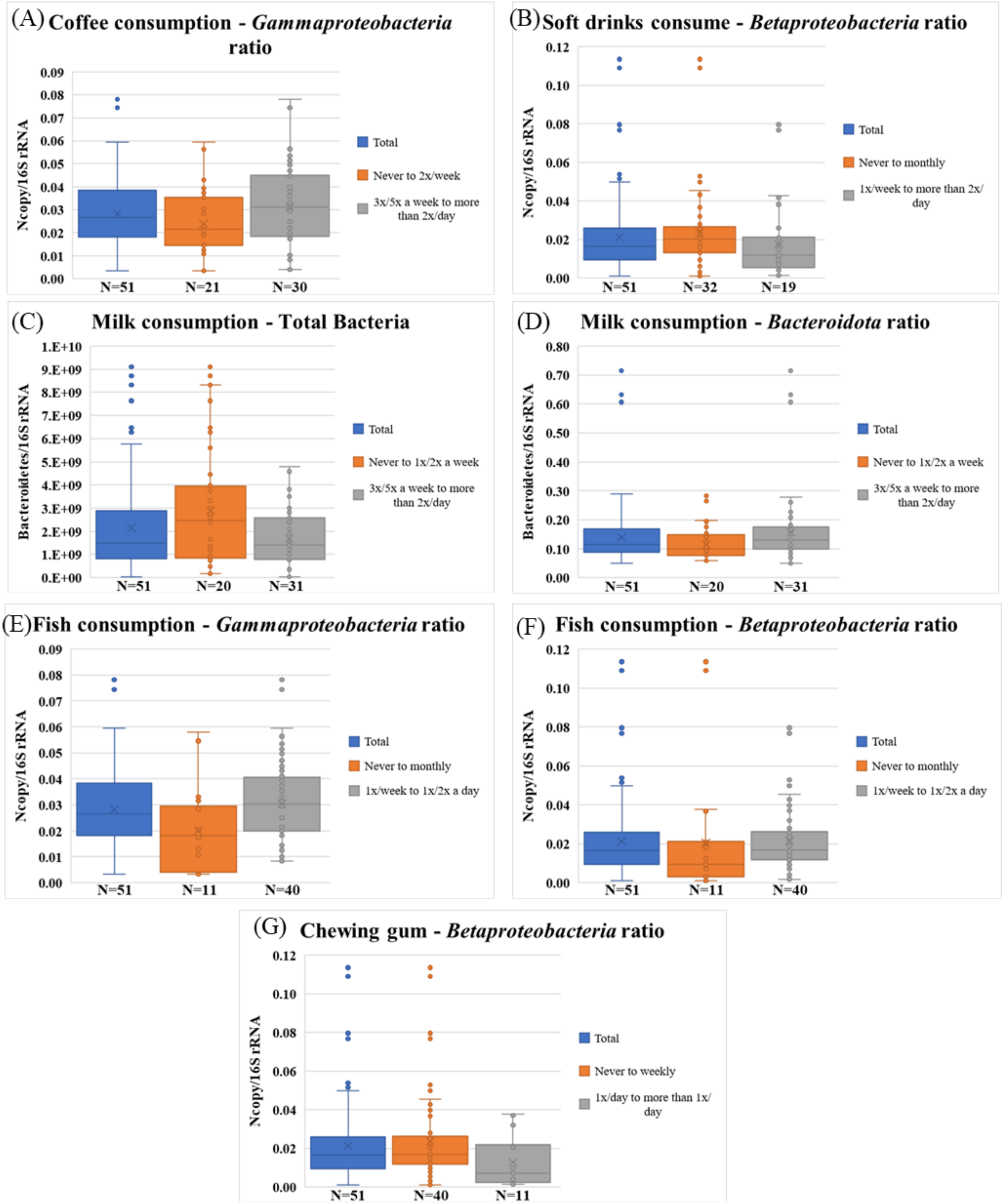


Figure 18 - Statistically different results for the diet habits: Box-plots show the different distribution of results obtained for the groups of individuals with different habits ($p<0.01$)– Coffee consumption and the (A) Gammaproteobacteria and (B) Betaproteobacteria prevalence; Milk consumption and the (C) Total bacteria abundance and (D) Bacteroidota prevalence; Fish consumption habits and the (E) Gammaproteobacteria and (F) Betaproteobacteria prevalence; (G) Cheqing gum and the Betaproteobacteria prevalence. The prevalence of bacterial groups was obtained normalizing the data (ncopy/ mL of sample) by the 16S rRNA quantification.

The diet habits of ingestion of coffee, soft drinks, milk, fish and chewing gum consumption presented significative differences in the ratios (taxon abundance / 16S rRNA gene) of *Gammaproteobacteria*, *Betaproteobacteria*, *Bacteroidota* and total bacteria. *Gammaproteobacteria* were slightly, although significantly less frequent in people who consume coffee up to two times a week (0.02 ncopy/16S rRNA) than in people who consumed at least 3 or 5 times a week (0.03 ncopy/16S rRNA). *Betaproteobacteria* was significantly more frequent in individuals who never consumed soft drinks or only consumed it monthly (0.023 ncopy/16S rRNA) than when they were consumed at least 1 time per week (0.018 ncopy/16S rRNA). Total bacterial abundance was significantly higher in people that consumed milk up to 1 or 2 times per week (2.9×10^9 ncopy/mL of sample) in comparison to people that consumed milk at least 3 times per week and up to more than 2 times in a day (1.7×10^9 ncopy/mL of sample*). Bacteroidota* were slightly less frequent in people that only consumed milk up to 1 or 2 times per week (0.11 ncopy/16S rRNA) than in people that had higher consume of milk (0.16 ncopy/16S rRNA). *Betaproteobacteria* were significantly more frequent in individuals that consumed never consumed chewing gum or consumed it weekly (0.02 ncopy/16S rRNA) comparingly to individuals that consumed chewing gum at least 1 time per day (0.01 ncopy/16S rRNA). *Gammaproteobacteria* were less significantly frequent in individuals that never consumed fish or consumed it monthly (0.02 ncopy/16S rRNA) than in people that consumed it at least 1 time per week up to 2 times per day (0.03 ncopy/16S rRNA). Concerning the *Betaproteobacteria* phylum, it was also less frequent, slighty but significantly in consumers of fish that never consume or have a monthly consume (0.020 ncopy/16S rRNA) in comparison to the individuals that consume fish at least 1 timer per week and up to 2 times per day (0.021 ncopy/16S rRNA).

Concerning the other habits in study, use of electronic cigarette, onychophagia condition and pen biting, significantly differences between the two groups of individuals were observed. *Gammaproteobacteria* were significantly more frequent in individuals that used electronic cigarette (0.05 ncopy/16S rRNA) when comparing to individuals that did not use electronic cigarette (0.03 ncopy/16S rRNA). Total bacteria abundance was also significantly higher in individuals that used the electronic cigarette (4.5×10^9 ncopy/mL of sample) than it was in individuals that did not used it (2.1×10^9 ncopy/mL of sample).

Gammaproteobacteria were more frequent in individuals that did not bite pens (0.03 ncopy/16S rRNA) than in individuals that bite them (0.02 ncopy/16S rRNA). For the other bacterial groups, a lower prevalence was observed in individuals that did not bite pens (0.7 ncopy/16S rRNA) in comparison to the ones that bite them (0.8 ncopy/16S rRNA).

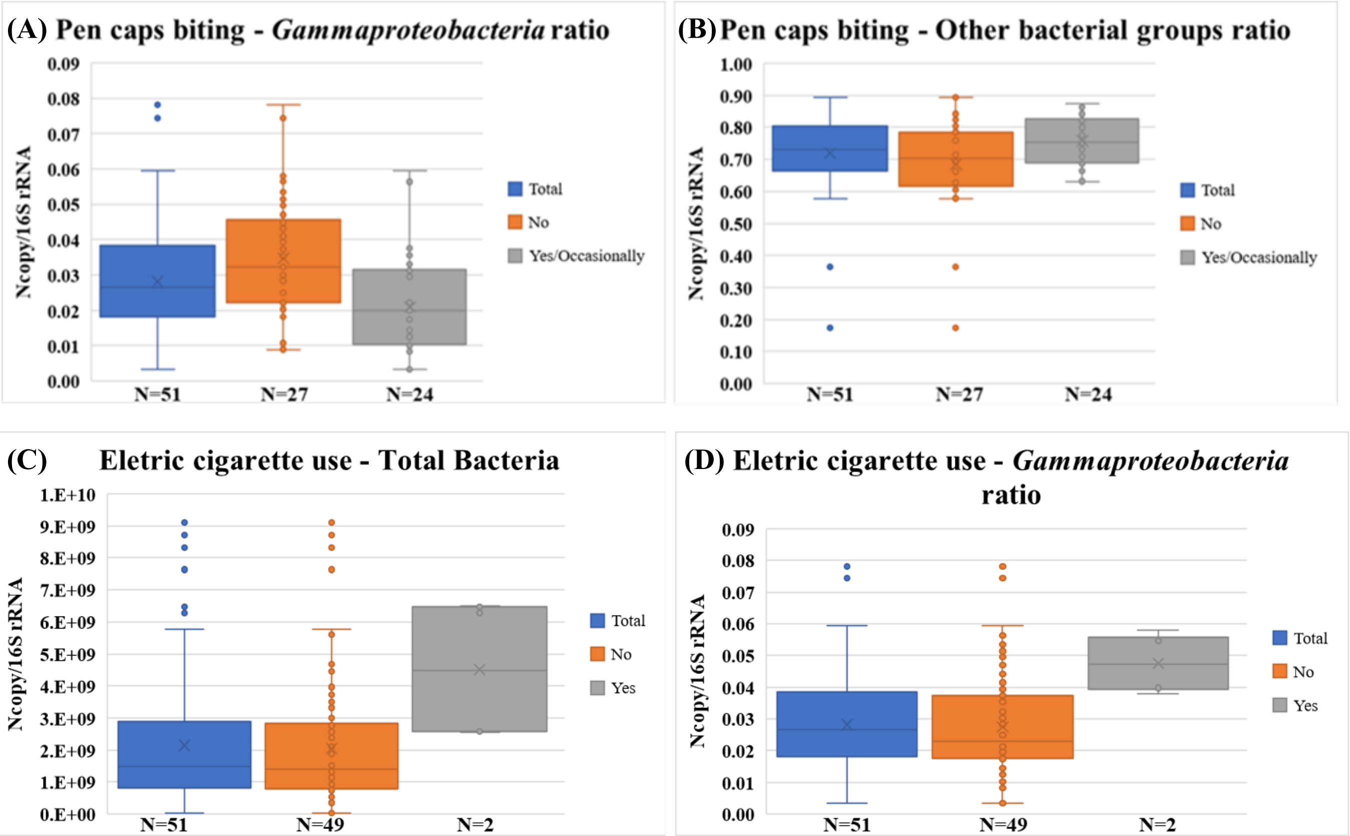


Figure 19 - Statistically different results for the other habits: Box-plots show the different distribution of results obtained for the bacterial groups that demonstrated significative differences (p<0.01) between groups of subjects – Pen bitting and the (A) Gammaproteobacteria and (B) Other Bacterial groups prevalence; Eletronic cigarette and the (C) Total bacteria abundance and (D) Gammaproteobacteria prevalence; The prevalence of bacterial groups was obtained normalizing the data (ncopy/ mL of sample) by the 16S rRNA quantification.

In the onychophagia condition, significantly differences between individuals that suffer from that condition and the ones that did not were observed for total bacteria abundance, *Actinomycetota*, *Bacillota*, *Gamma*- and *Betaproteobacteria* and in the Other bacterial groups. In terms of total bacteria abundance, individuals that suffer from the condition presented higher abundance (3.3 x 10^9 ncopy/mL of sample) than individuals that did had onychophagia (1.9 x 10^9 ncopy/mL of sample). *Actinomycetota* were significantly more frequent in individuals that did not had onychophagia (0.09 ncopy/16S rRNA) than in the ones that had it (0.04 ncopy/16S rRNA). *Bacillota* were also more frequent in people that did not suffer from onychophagia (0.014 ncopy/16S rRNA) in comparison to people that suffer from it (0.010 ncopy/16S rRNA). *Gammaproteobacteria* were significantly more frequent in individuals that did not had onychophagia (0.03 ncopy/16S rRNA) than in people that did not had it (0.02 ncopy/16S rRNA). Betaproteobacteria also followed the trend, in terms of prevalence, of the previous taxonomic groups. Betaproteobacteria was more frequent in individuals that did not had onychophagia (0.021 ncopy/16S rRNA) in comparison to individuals that did not had it (0.020 ncopy/16S rRNA). For the Other bacterial groups, individuals that suffer from onychophagia presented a higher prevalence (0.8 ncopy/16S rRNA) in comparison to the ones that did not suffer from it (0.7 ncopy/16S rRNA).

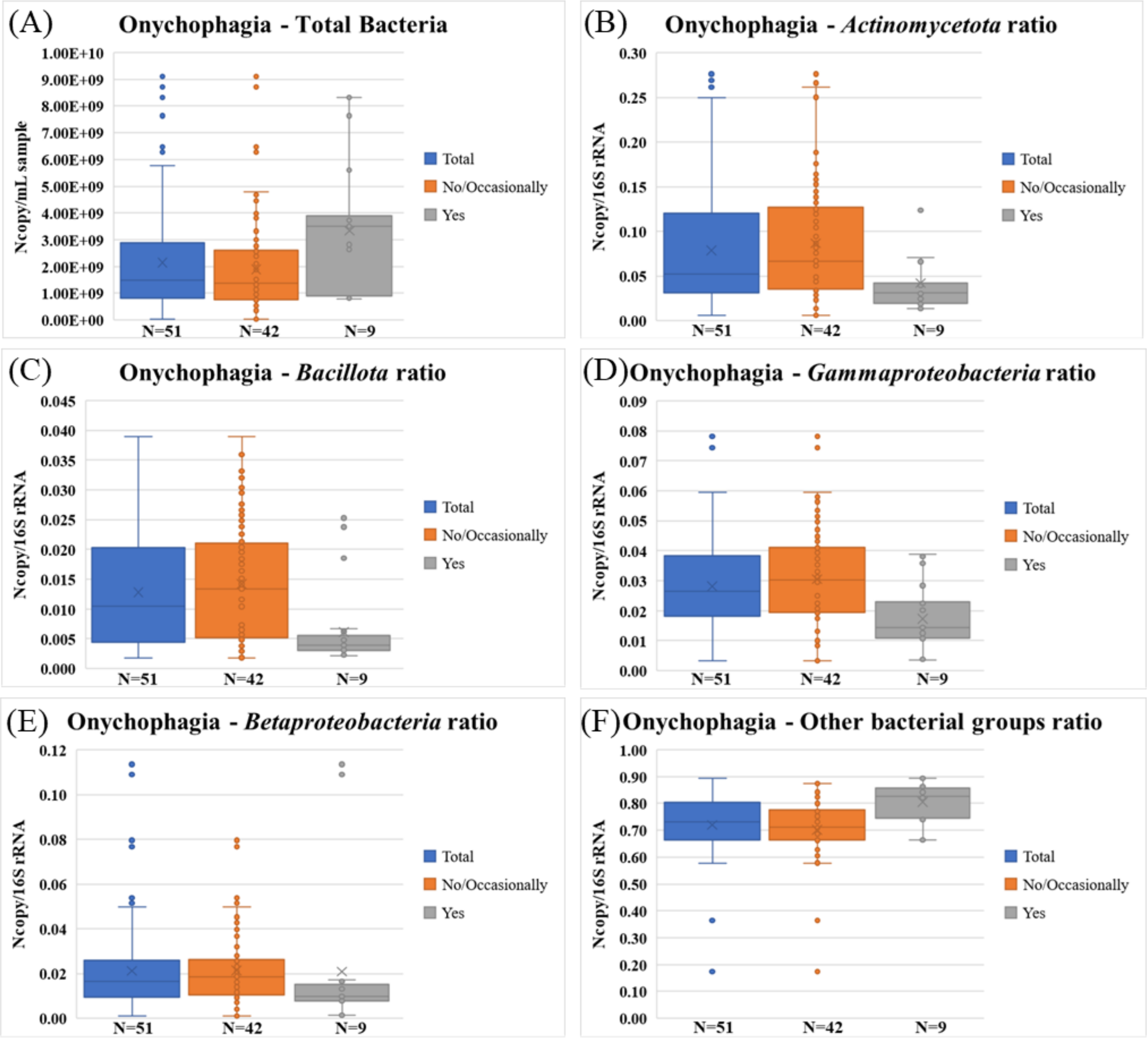


Figure 20 - Statistically different results: Onychophagia – Box-plots show the different distribution of results obtained concerning the condition of onychophagia. The bacterial groups that demonstrated significative differences are showed (p<0.01) – (A) Total bacteria abundance; (B) Actinomycetota prevalence; (C) Bacillota prevalence; (D) Gammaproteobacteria prevalence; (E) Betaproteobacteria prevalence; (F) Other bacterial groups prevalence. Total bacteria abundance was measured by the quantification of the 16S rRNA gene. The prevalence of bacterial groups was obtained normalizing the data (ncopy/ mL of sample) by the 16S rRNA quantification.

Assesing possible correlations between bacterial groups´prevalence and individuals´ variables

Possible correlations between the prevalence of the bacterial groups and the different individual’s variables were assessed based on a multivariate analysis (Figure 20). Total variation of the samples was 359.157 and only 17% could be associated with the explanatory variables along axis 1 and 8% along axis 2. The explanatory variables selected from 19 that were tested, were sex (p=0.001), chewing gum (p=0.001), Carious, lost and clogged teeth (CPO) index (p=0.001), antibiotic intake (p=0.001), onychophagia (p=0.008) and pen caps biting (p=0.008). As can be observed by the size and angle of the arrows of the explanatory variables (Figure 21), the first four explain most of the variation of the samples.

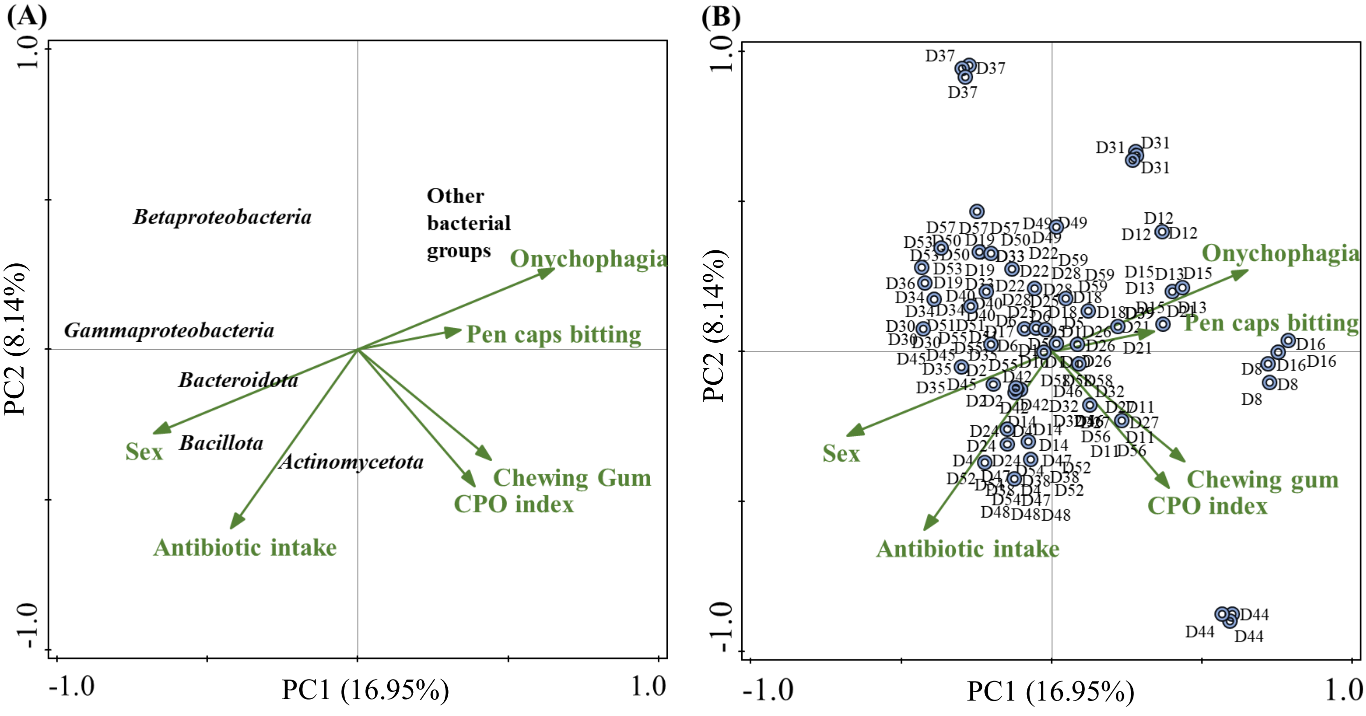


Figure 21 - Redundancy analysis (RDA) of the variation of prevalence of bacterial groups in saliva samples in function of lifestyle habits. (A) - Test variables and explanatory variables; (B) – Samples and explanatory variables. The test variables (prevalence of the bacterial groups Actinomycetota, Gammaproteobacteria, Betaproteobacteria, Bacteroidota, Bacillota and Others bacterial groups) are represented in black and the explanatory variables in green. The explanatory variables were forward selected if presented a p value <0.01. The explanatory variables were associated with 28.3% of the observed variation among the test variables.

Analysing the figure, it is observed a positive correlation of the ratio of *Bacillota*, *Bacteroidota*, *Actinomycetota* and *Gammaproteobacteria* groups with the explanatory variables sex and antibiotic intake in the previous 12 months to the beginning of the study ($p<0.01$). The ratio of the *Betaproteobacteria* group is negatively correlated with the CPO index and chewing gum consumption ($p<0.01$). Positively correlated to the explanatory variables onychophagia and pen caps biting is the "others" bacterial groups ratio.

It is also possible to observe that chewing gum, biting nails or pens might be related to the decrease of the bacteria from the *Bacteroidota, Gammaproteobacteria, Bacillota* and *Actinomycetota* phyla.

Also associated with the explanatory variables chewing gum and CPO index is the separation of sample D44 from the others. Sample D12 and D31 correlated positively with the prevalence of the "others" bacterial groups.

Discussion

The oral microbiota is distributed by different places in the oral cavity, and the objective of this study was to have an overview of the whole cavity microbiota. Hence, the method of acquisition of saliva samples was a good compromise between sampling easiness and adequacy to the study objectives. Indeed, considering that saliva contacts with the whole cavity and sites inside it, it is possibly the most representative type of oral sample of the oral cavity (Fakhry et al., 2018). A major strength of this collection method is that it is easy to perform. In comparison with other methods, for example collection by drooling, the collection method applied in this study is easier to perform and fast, as it can take only 5 minutes. Studies comparing different types of oral samples collection methods concluded that the saliva microbiota and microbiome profiles are not significantly affected by it, presenting similar results (Lim et al., 2017; Omori et al., 2021).

In this study we focused on *Actinomycetota, Gamma-* and *Betaproteobacteria, Bacteroidota and Bacillota* phyla because they represent the most abundant phyla and classes and we were interested in assessing if their variations could be influenced by external variables as different studies already proposed (Belstrøm et al., 2014; Cornejo Ulloa et al., 2019; Gao et al., 2018b; Nasidze et al., 2011). The qPCR sensitiveness was considered an added value for this purpose. The literature available suggests that total bacteria abundance in these type of samples range from 2.0 x 108 copies of the 16S rRNA gene to 35.7 x 108 copies of the 16S rRNA gene per mL of sample (Lee et al., 2022) and a mean value of 13.56 x 10^8 copies of the 16S rRNA gene per mL of sample in non-smoking, with lesser than 2 lost teeth, healthy adults. The mean obtained in the present study demonstrated to be higher than that, 2.2×10^9 copies of the 16S rRNA gene per mL of sample of total bacteria abundance.

However for the DNA concentration, it is not possible to correctly conclude if the results obtained are in agreement with the existing literature, due to the existence of a large range of values (190 ng DNA to 667 ng DNA)(Poetsch et al., 2022).

Gammaproteobacteria and *Betaproteobacteria* are the *Pseudomonadota* classes more relevant for the oral cavity (Leão et al., 2023), and were quantified in this study. According to the literature analysed and described in the introduction section, Table 1 and Table 2, the results obtained show the same trend, *Gammaproteobacteria* presenting a higher abundance than *Betaproteobacteria* class. For the remaining bacterial groups, the results obtained are not similar to the results observed in the existing literature. The groups results can be organized like this, by increasing abundance: *Bacillota* - *Betaproteobacteria* - *Gammaproteobacteria* - *Actinomycetota* - *Bacteroidota*. While it was expected to look like this: Actinomycetota > Bacteroidota > Pseudomonadota > *Bacillota* (Deo & Deshmukh, 2019; Dewhirst et al., 2010; Verma et al., 2020).

The questionnaire applied to the participant students was comprised of 68 questions. Only 19 out of these were considered for the study. Although being interesting topics - stress status (Martínez et al., 2022; Paudel et al., 2022), breastfeeding period (Butler et al., 2022; Timby et al., 2017), type of birth (Li et al., 2020) and exchange of saliva with other human beings (Kort et al., 2014), for example – it was not possible to analyse all the topics being the 19 topics selected due to being considered as more relevant accordingly to the existing literature.

The transformation of the responses into a binary matrix might have also affected the results profiles and statistical analyses. The criteria used aimed to group answers that reflected identical behaviors. For example, in the habit “daily toothbrushing”, the possible answers in the questionnaire were “less than 1x per day”, “1x per day”, “2x per day”, “3x per day”, “4x per day” and “more than 4x per day”. In terms of amount of time, “less than 1x per day”, “1x per day” and “2x per day” are the expected for most cases. Therefore, they grouped in “0” and the others in “1”. However, if we changed this criterion, the results might have been different.

The different age groups presented a similar behaviour for the taxonomic groups in study, however showed different results for total bacteria abundance. This can suggest a period, between the age of 18 to 21 years, where the similar lifestyle – college student lifestyle – leads to the shaping of the oral microbiome to being similar for subjects within these ages.

The analysis of the results obtained for the sex characteristic needs a more cautious analysis since both sexes were not equally represented. The male sex presented higher total abundance and higher prevalence of the “others” bacterial group. This suggests that the male subjects, might have present in the oral cavity other relevant and noticeable bacterial groups. This is in accordance with Minty et al., (2021) and Kim et al., (2020) studies, who showed that males had higher bacterial diversity than females in oral cavity and the gut. Since there is an oral cavity – gut axis, and an increasing number of studies report that these have a close relationship and that dysbiosis situations in the oral cavity also affect the gut (Lam et al., 2022; F. Liu et al., 2022; Park et al., 2021), it is normal to assume that the oral cavity microbiota is different between male and female.

The literature suggested that although some weeks may be necessary after antibiotic intake for the oral microbiota to reach a new state of equilibrium (Elvers et al., 2020), there were still observable differences between the study and control groups that had took the antibiotics. In this study, differences were observed for *Actinomycetota* and *Bacillota* phyla (Figure 16), having the samples from the groups that did take antibiotics higher prevalence. This could suggest that due to possible antibiotics tolerance (amoxicillin, phosphomicin, penicillin) and alterations in the oral microbiome during the time of effect, bacteria belonging to these two phyla thrived. Less competition with other bacteria affected by the antibiotic or less resistant to it, alterations to the environment due to medication intake among other factors must be accounted to explain these results. These significant differences are in accordance with the existing literature that the effect of antibiotic use or other disease might enable the increase of some taxonomic groups prevalence (Arredondo et al., 2020; Y. Wu et al., 2021). To further understand these results, it would be interesting to perform a microbiota analysis of the oral cavity of healthy, adult subjects, within the same age group before and after use of antibiotics to check these differences and understand if, for example, results just like the ones obtained might correspond some intermediate equilibrium state. There are little studies concerning the effect of antibiotic intake in the oral cavity microbiota. To better understand the impact of these medicine, more studies using qPCR or even metagenomics should be performed.

Teeth brushing aims to lower the availability of nutrients to microbes and the physical removal of those colonizing the oral cavity. However, no important differences were observed, probably due to the categories generated for the statistical analysis. Interestingly, for individuals reporting toothbrushing at least 3 times a day, it was observed a significant lower prevalence of "other" bacteria. *It is hypothesised that the groups in study, Actinomycetota (Actinomyces* spp.), *Bacillota (Streptococcus* spp*.), Gammaproteobacteria (Haemophilus* spp*.), Bacteroidota (Capnocytophaga* spp*.)* and *Betaproteobacteria (Neisseria* spp*.)* may attach to the oral surfaces to initiate the formation of biofilms, being less affected by normal toothbrushes (Huang et al., 2011). The use of electric toothbrush has been demonstrated to aid to manage the plaque formation and to have a better performance in eliminated the biofilms in the oral cavity (Zautner et al., 2013). In our study, the users of electric toothbrush presented significative lower prevalence of *Betaproteobacteria* and higher of *Bacteroidota*, than the users of normal toothbrushes. While the removal of *Betaproteobacteria* with electric toothbrush, it is possible that other microbiota community members, such as *Bacteroidota*, may overgrow. Nevertheless, the possibility of contamination of this type of toothbrush and the impact that this can have in the oral cavity microbiota (Zautner et al., 2013; Zúñiga et al., 2022), may deserve further investigation. Other cleaning tool analyse and respective effect on the oral microbiota was the dental floss that also presented a reduction in the prevalence of "others" bacterial groups. Dental floss would affect specially the bacteria that prefers the small gaps between teeth. These communities, due to the type of sample and sampling procedure, might have not been correctly represented in the study.

The chewing gum created in 1840s, has now a wide range of uses including, to improve the breath odour, after a meal. Although not quite explored, chewing gum was proved to collect and enable the elimination of microorganisms in the oral cavity, at least for a short period of time (Takeuchi et al., 2018), being quantified 10^8 bacteria in the study developed by Wessel et al., (2015). A more rapid clearance and cleaning of the oral cavity and sites is achieved by chewing gum considering that will lead to an increase in salivation and consequent higher flow rate of saliva. This cleaning leads to a decrease in the availability of nutrients and other important factors for the bacterial growth and colonization (Aravinth et al., 2018). Concerning these aspects, it would be expected the detection of statistically differences for total bacterial abundance between subjects that normally chew gum never to weekly and 1 to more than 1 time per day. However, only for the relative abundance of *Betaproteobacteria* were observed significant differences, with lower prevalence in the chewing gum user group. Possibly differences might have been observed for other groups if other dichotomization criterion was used, for instance if users were considered those that consumed chewing gum more than once a day. This topic is interesting as chewing gum, especially containing xylitol, may be a suitable agent to improve hygiene and oral health (Inchingolo et al., 2022).

Nail biting and biting the cap of pens is common in children and young adults, and may be associated to boredom, frustration, and stress. Onychophagia is described by Chinnasamy et al., (2019) as "an oral compulsive behavioural disorder of nail biting". Despite the scant literature tackling this them e, it might be expected an increase of *Gammaproteobacteria* since *Enterobacteriaceae.* presented a significant increase in the studies analysed (Chinnasamy et al., 2019; Kamal & Bernard, 2015). For subjects suffering from this condition there was higher total bacteria abundance and prevalence for the "Others" bacterial groups. Curiously, it was observed a decreased prevalence for the *Actinomycetota*, *Bacillota*, *Gammaproteobacteria* and *Betaproteobacteria* groups. The prevalence for the "Others" bacterial groups, in the pen caps biting, was also the one that showed an increase in comparison with the group of subjects that did not bite the caps of pens. The *Gammaproteobacteria* class showed lower prevalence. These possible unexpected results, for both characteristics, might be connect to the effect that chewing might have in the mouth and the process it activates (more salivation, production of enzymes, among others). The chewing effect can possibly lead to a harsher environment in the oral cavity for the development of bacteria, explaining the prevalence decreases verified (Al-Manei et al., 2020). The increase in the "others" bacterial group might be due to environment contamination of the objects, in this case, pens.

The literature reports a decrease in members of the phylum *Pseudomonadota* (Sato et al., 2020; Weyrich, 2021) in smokers. In this study only two people were smokers, which hinders any solid conclusions. Chopyk et al., (2021) demonstrated that electric cigarettes (e-cigarettes) users had a bigger diversity in saliva and buccal samples comparing to a control group of non-smokers or non-vapers smokers. The abovementioned study also demonstrated an increase of the genera *Veilonella* (*Bacillota* phylum) and *Haemophilus* (*Gammaproteobacteria* class). In the study developed in the current study, we also observed a higher prevalence of the class *Gammaproteobacteria* of e-cigarettes users. Although the results agree for this class, the conclusions to take from this must consider that the sample size of electronic cigarettes was 2, so further studies should be performed applying this technique to confirm the results obtained and check possible differences for the *Bacillota* phylum. The study of Chopyk et al. (2021) is interesting as it compares to the present results with a study population also comprised of college students, 75% of which were females and the sample size was 52 subjects. The main difference is that here the qPCR technique was used while in the literature referred applied 16S rRNA amplicon sequencing through Illumina and pyrosequencing (Table 1).

In terms of diet habits, there was not statistically difference observed for meat consumers and non-consumers. Hansen et al. (2018) observed an increased prevalence of genera belonging to *Pseudomonadota* and *Bacteroidota* in vegan individuals, however the results we report here for meat and fish consumption are contradictory to this since no significantly differences were observed between the two types of meat consumers and in the fish consumption *Gamma*- and *Betaproteobacteria (Pseudomonadota)* demonstrated higher prevalence in individuals that consumed fish between 1 time per week up to 2 times day. Coffee, soft drinks and milk consumption was also studied. In a previous study concerning coffee consumption no significant differences were detected in the oral cavity microbiota (Peters et al., 2018). Peters et al., (2018) used oral wash samples, a fact that may explain the differences, since saliva samples are more concentrated and may be more sensitive to study microbiota variations. The decrease of *Betaproteobacteria* observed in subjects that consumed more soft drinks can possibly be explained for the effect that some soft drinks, for example Coca-Cola, have in the oral pH (De Lima Almenara et al., 2016). Since that some soft drinks can lower the pH of the oral cavity, and *Betaproteobacteria* does not present a positive correlation with environments more acidic (Yun et al., 2016), it is possible to conclude that the decrease of this bacterial class is due to this alteration in the oral environment. Considering the above discussion and the explanatory variables listed based on the Redundancy Analysis (figure 20), it is possible to suggest that some factors may have a synergistic or antagonistic effect on the oral microbiota. The sex of the individual, chewing gum consumption, CPO index, antibiotic intake before the study, onychophagia and pen caps biting were listed as explanatory variables. From these the CPO index did not show significant differences between the two subject groups.

Sex, age, antibiotic intake, daily toothbrushing, dental floss, use of electric brush, coffee, milk, soft drinks and fish consumption, electronic cigarette use, onychophagia, pen biting and use of chewing gum demonstrated significant differences between groups of individuals. However in the RDA performed, to assess possible correlations the same effects of the certain variables (sex, antibiotic intake, CPO, use of chewing gum, onychophagia and pen biting) were observed in the taxonomic group prevalence's presenting the same effect on samples as previously show in the study (Table 11).

Subjects participating in the study had taken the antibiotics at least two months previously to sample collection but differences were still found. The onychophagia and pen caps biting have almost non existing literature to compare the results. Chewing gum had some contrary to the literature results. However, with the theoretical literature and the limited experimental literature available, the explanatory variables that demonstrated differences between the groups might be true oral microbiota shaping factors. It is also relevant to mention the possibility of some of the variables used, being confounding variables which would also biased the discussion of the correlations obtained.

With the increasing use of saliva samples and oral cavity samples in the diagnosis field, standardized methods for the collection, processing, treatment, DNA extraction and storage of samples is still missing. However, more studies concerning the composition of the microbiota of the oral cavity of healthy human adults are needed (D'Amone et al., 2021; Omori et al., 2021).

The effect and importance of the human microbiota in the general health status and the possible applications, such as, subject oriented health treatments and the use as a non-invasive disease diagnostic tool are one of the possible applications of this type of studies. The study of oral microbiota and their metabolites, genes being expressed and other information concerning the microbiota can also give new information concerning the systemic diseases and the dysbiosis on the oral cavity, enabling the possibility to start treating a disease before having symptoms (Barbour et al., 2022; Belibasakis et al., 2019; Bessa et al., 2022; Cai et al., 2022).

Conclusions and Future Work

Conclusions

The characterization of major phyla and classes in the oral cavity of young adults showed a conservative profile within the group and in agreement with what is described in the literature.

Intrinsic features (age, sex) and external factors (diet, hygiene, etc) were observed to be associated with microbiota composition.

The binary categorization of the variables and the interplay among them may hinder an accurate evaluation of factors potentially affecting the oral microbiota. A larger population and alternative categorization may contribute to improve the output of this study.

Quantitative PCR, being more sensitive and offering a quantitative output is an adequate technique for targeted analyses of microbiota, as was demonstrated in this study. However, primer design, and sample collection and processing would need to be improved and standardized between studies.

Future Work

This study was initiated with the goal of detecting antibiotic resistance genes and mobile genetic elements in saliva samples. This was the reason to use quantitative PCR for the analysis. These analyses will be essential to complete this study.

From this work, it would be important to perform the 16S rRNA amplicon sequencing in the DNA present in the samples to verify the results obtained but also to compare results of microbiomes with the results concerning the oral cavity microbiota that already exist in the literature and are mainly metagenomic/microbiome results.

In the future, it would be interesting to perform this same study in a subject group more random, that could better represent the Portuguese adult population to have more confidence in the results and to decrease the possible bias related to the subject group, perform it with a bigger sample size. If age groups were well represented, it would be a great step to also perform a horizontal study.

Developed qPCR protocols to other bacterial groups would also be interesting, especially considering the great abundance of "others" bacterial groups detected in this study. It would be also beneficial to then apply those protocols to other sample types, clinical and environmental.

The final possible future steps would be to create primer pairs from the scratch to compare the efficiencies and results with the ones obtained from pre-existing primers.

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