CATÓLICA
INSTITUTO DE CIÊNCIAS DA SAÚDE
LISBOA · PORTO · VISEU

”ORAL MICROBIOME AND HOST HEALTH: IS THE FIRMICUTES/BACTEROIDETES RATIO AN INDICATOR OF ORAL AND SYSTEMIC HEALTH?”

Dissertação apresentada à Universidade Católica Portuguesa
para obtenção do grau de mestre em Medicina Dentária

Por:
Sara Sofia Dinis de Sousa

Viseu, 2017

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Orientador: Doutora Maria José Correia
Coorientador: Doutora Marlene Barros

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Epigraph

“It is the supreme art of the teacher to awaken
joy in creative expression and knowledge.”
- Albert Einstein

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Resumo

Introdução: A saúde oral é determinada por vários fatores e pode influenciar e ser influenciada pela saúde sistémica. Na população idosa as questões de saúde oral são de particular importância e as necessidades de tratamento são mais prevalentes.

O objetivo deste estudo foi estabelecer uma estratégia multidimensional que visa a caracterização da saúde oral de uma população de idosos da região de Viseu, sob vários aspetos, desde os indicadores do estado de saúde oral, até às características bioquímicas e microbiológicas da saliva, passando pela associação com patologias sistémicas. Outro objetivo foi estabelecer a metodologia para quantificação de *Firmicutes* e *Bacteroidetes* em amostras de saliva por tecnologia RT-PCR no laboratório SalivaTec.

Métodos: Neste estudo foram usadas três estratégias: questionário para recolha de dados socio-demográficos e sobre a autoperceção da saúde oral; avaliação clinica da mesma e recolha de saliva para estudar parâmetros bioquímicos e microbiológicos.

Resultados: Os CPODs observados foram 20,82 e 78% da população em estudo apresentava periodontite (PSR 2-4). A proteína 14-3-3 sigma é proposta como biomarcador de estratificação de indivíduos com patologia periodontal e co morbilidades. Ao analisar a carga total bacteriana observou-se uma quantidade de *Firmicutes* superior (20%) à quantidade de *Bacteroidetes* (3%) e o rácio F/B médio foi 12,84. Os índices bacterianos não diferiram de forma estatisticamente significativa entre as várias estratificações realizadas.

Discussão: Os resultados indicam que a saúde oral da população idosa de Viseu pode ser melhorada principalmente na diminuição do número de dentes perdidos e na melhoria da saúde periodontal. Não houve diferenças significativas nas associações entre fatores bioquímicos e microbiológicos da saliva e a saúde oral. Verificou-se discrepância entre indicadores de saúde oral avaliados clinicamente e a auto-percepção.

Conclusão: Esta tese mostra uma estratégia multidimensional para a avaliação da saúde oral na população idosa de Viseu.

Palavras-chave: saliva; saúde oral; saúde sistémica; séniores; microbioma

Abstract

Introduction: Oral health is determined by several factors including systemic health. In the elderly oral health issues are prevalent and treatment needs increased. The goals of this work were to stablish a multidimensional strategy to characterize oral health in an elderly population of Viseu in different dimensions from oral health indicators, to salivary properties including microbiome evaluation. A secondary objective was the establishment of the laboratory procedures for *Firmicutes* and *Bacteroidetes* quantification by RT-PCR technology.

Methods: In this study 3 strategies for data collection were used: questionnaires for sociodemographic data and self-perceived oral health; clinical assessments of oral health determination and saliva for biochemical and microbiological parameters.

Results: DMFT indices we 20.82 and 78% of the population presented with periodontal disease (PSR 2-4). 14-3-3 protein sigma is proposed as a stratification biomarker for individuals with periodontal disease and comorbidities. Salivary bacterial analysis demonstrated that *Firmicutes* (20%) are more prevalent than *Bacteroidetes* (3%) and the mean F/B ratio was 12,84. Bacterial indices were not statistically different in the different sub groups.

Discussion: Results indicate that this populations oral health is may be improved especially regarding missing teeth and periodontal status. There were no statistical differences in the association between biochemical and microbiological parameters and oral health. There were differences in the clinically assessed oral health levels and the self-perceived oral health.

Conclusion: This thesis provides a multidimensional strategy towards the evaluation of the oral health of a senior population in Viseu.

Keywords: saliva; oral health; systemic health; seniors; microbiome

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Abbreviations list

DMFT index	Decayed, Missing and Filled Teeth index
PSR index	Periodontal Screening and Recording index
WHO	World Health Organization
PD	Periodontal disease
GOHAI	Geriatric Oral Health Assessment Index
qPCR	Quantitative Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
F/B	*Firmicutes*/*Bacteroidetes*

Introduction

Introduction

The elderly represent an age group in which medical care treatment is more frequently requested and needed. In addition, as the average life expectancy grows, so do chronic conditions such as cardiovascular or neurological diseases (1,2). From ailments affecting elderly populations, oral health conditions are amongst the most frequent (3). Intervention in this health determinant is commonly based on the development of effective oral health promotion strategies. Furthermore, it is important to evaluate the impact of oral diseases on the systemic health and quality of life in the elderly.

The World Health Organization (WHO) created the policy framework for “Active Aging” in order to optimize opportunities for health, participation and security and improve quality of life as people age.

There is evidence that longevity and quality of life can be improved by adopting healthy lifestyles including the participation in physical activities, having a healthy diet, not-smoking or having non-alcoholic habits and responsibly taking the prescribed medication (4,5). Acknowledging this fact, many local entities have promoted physical activity programs focused on the elderly population. Our target population is from Município de Viseu Portugal, integrated in the “Atividade Senior” program that promotes healthy life styles for citizens over 55 years old. The Universidade Católica Portuguesa through the Instituto de Ciências das Saúde - Viseu, specifically the SalivaTec laboratory, has been a partner in this program since 2016 by studying salivary biomarkers and oral health of the participants. This thesis is integrated in this partnership.

Geriatric health problems are associated with polypharmacy (6,7), escalation of acute disorders to chronic conditions and social and environmental factors (8). Furthermore, psychosocial changes with age, such as, emotional aspects, anxiety, depression, cognitive function and alcoholic and smoking habits are known to affect oral health (9). Therefore, a multidisciplinary approach is essential to effectively monitor and understand the overall health status of these individuals (9).

1. Geriatric oral health

Oral disease, despite being largely preventable, remains the most common chronic disease worldwide and has a significant negative impact on quality of life, particularly among older adults (10).

1.1. Dental caries

The most prevalent oral diseases among the general population are dental caries and periodontitis (5,11,12). The WHO proposes several oral health indicators such as: number of caries, number of lost and treated teeth which may be expressed as the Decayed, Missing and Filled Teeth (DMFT) index (4). DMFT is well-established as the main measure of caries experience in dental epidemiology assessment (4,13,14).

In Portugal, there are a few studies assessing oral health status in populations other than children and adolescents (14–17). That is one of the reasons why we are investing our efforts specifically in the elderly population, which not only have been increasing in numbers and are a major part of the population in this region, but as mentioned before, are a population which frequently needs treatment. In 2015, the Ordem dos Médicos Dentistas (OMD) which is the Portuguese National Board regulating the Dental Medicine Profession, conducted the III National Prevalence Study of Oral Diseases covering several health indicators and lifestyle habits. This study found that DMFT index was 15,11 (decayed teeth 1,57; missing teeth 11,44; filled teeth 2,10) for the Portuguese elderly population (17). Furthermore, the population over 65 years of age showed 1,1% carie free teeth and 14,4% toothless subjects (17). A Brazilian study in an elderly population showed a higher DMFT index of 29,24 (18). Nevertheless, an elderly population from China showed a lower DMFT index (13,90 ± 9,64) than the Portuguese population (19).From studies in 22 countries, only India (DMFT=11,29 ± 8,37) (20) and China (19) showed a lower DMFT mean comparing to the Portuguese elderly population. This indicates that the Portuguese elderly population DMFT is better than the majority of the elderly population from New

Zeland (DMFT=19) (21), Mexico (DMFT=17,2) (22), Denmark (DMFT=22) (23), and others.

Moreover, studies from Italy (24) and from Sweden (25) showed a higher tooth loss among females comparing to males which is also registered for the Portuguese population (17). These data suggest that although oral health regarding caries levels in Portugal seems to be above that of other countries, there is still room for improvement and more importantly there is the need for a wider evaluation of the elderly population.

1.2. Periodontal health

It is predicted that 20-50% of the population around the globe have periodontal disease (11). The evaluation of periodontal status is often based in Community Periodontal Index (CPI) which determines periodontal damage and verifies the need for periodontal treatment (26), and Periodontal Screening Record (PSR) that defines the periodontal severity by sextants (15). The WHO keeps an updated data base of the periodontal disease distribution by country and by age range, using CPI (27). It is noteworthy that there are no data for Portugal in such database referring to adults over 65 years old. The data presented are for adolescents (15yo) and adults (35-44) and refer to 1989 and 1984 respectively. It should be reinforced that this WHO database was last updated in January 2017.

Studies by Calado *et al.* (2015) estimated a CPI of 29,5% periodontal healthy, 39,4% bleeding on probing, 12,3% 4-5 mm periodontal pockets and 3,0% ≥6 mm periodontal pockets (17), which seems to indicate that periodontal disease is rather prevalent in the Portuguese elderly. Concerning other countries, a Brazilian study for an elderly population assessed a CPI of 19% periodontal healthy, 2% bleeding on probing, 44% presented calculus, 26% 4-5 mm periodontal pockets and 9% ≥6 mm periodontal pockets (27). Regarding the same age range, a study from China showed CPI of 0% periodontal healthy, 0% bleeding on probing, 39% presented calculus, 33% 4-5 mm periodontal pockets and 28% ≥6 mm periodontal pockets (27) which reveals a much worse situation than in Portugal. According to the WHO database, for the elderly population, only two countries showed a higher percentage of periodontal healthy individuals when

compared to the Portuguese elderly (29,5%) (17): Hungary 44% and Slovakia 63% (27). Also, few countries, namely, France, Hungary, Madagascar, Taiwan and Turkey, had a 3-4% ≥6 mm periodontal pockets, similar to the Portuguese data, all other countries show a higher prevalence of the highest periodontal score (27). These data suggest that although periodontal health levels in Portugal seems to be below those of Hungary and Slovakia, compared to countries such as Cambodia, Chile, China, Croatia, Estonia, Hong-Kong, India, Japan, Myanmar, Eslovenia and Turkmenistan (where reports indicate an absence of individuals aged 65 or more with an healthy periodontum), Portuguese elderly show better periodontal health.

Periodontitis is a multifactorial disease that includes bacterial and viral colonization and an inadequate immune response (28). Several factors are known to affect this condition, such as genetics, socialization, oral hygiene, as well as, smoking and alcoholic habits (28,29). Periodontitis is linked to several systemic diseases including cardiovascular, cerebrovascular and diabetes mellitus (30,31).

Although some of the factor affecting periodontal health are unmodifiable, it is known that regular dentist visits, oral health education and oral hygiene may improve the periodontal status of the individual (32). Therefore, it seems important to have a knowledge of what is the specific oral health status regarding periodontal disease of this population is in order to better adjust the oral health interventions to be done.

1.3. Oral and systemic health

Oral health has a close relationship with systemic disease (12,33–36). A poor oral health is known to represent a risk factor to develop systemic diseases (12). Oral health affects the systemic health in two ways: i) inflammation caused by periodontal problems; and ii) caries and other conditions that can lead to tooth loss which is associated with mastication and nutritional dysfunctions (12). Furthermore, elderly are more prone to xerostomia, orofacial pain, and oral cancer (37), as well as, other benign conditions of the soft tissues such as candidiasis, erythematous lesions (stomatitis) and angular cheilitis (38).

In periodontal disease there is a local response of the host to periodontal pathogens which results in the formation of an intense inflammatory infiltrate (12).

In spite of not knowing, the underlying mechanisms in most cases, the systemic host inflammatory response seems to explain the association between periodontitis and systemic diseases such as diabetes (39), cardiovascular disease (40), dementia/cognitive impairment (41) or more specifically Alzheimer's Disease (42) reported by some studies. Although some of the results from these studies are not conclusive, it is suspected that the mechanisms which might explain these associations are related to 3 main events: i) presence in the circulatory system of molecules such as pro inflammatory cytokines which produce a low grade systemic inflammation with vascular and other repercussions; ii) bacterial invasion or bacterial products present in the circulation and other host tissues and iii) the vascular mechanisms elicited by the presence of periodontal pathogens in the circulatory system which result in platelet aggregation and atheroma or small thrombus formation (43).

Functional issues, like chewing problems are relate to nutritional issues (12). Poor oral health status is one of the most frequent causes of malnutrition which can lead to severe deficiencies in energy and nutrient intake (12). This fact is related to a compromise of important functions of the oral system such as mastication and swallowing. Studies in the UK (44) and US (45,46) show an inverse relationship between poor dental state and adequate dietary intake with tooth absence being a clear risk factor. Clearly dental status is not the only variable influencing food intake and factors such as socio-economic status, general state of health and degree of dependence, but it seems to be rather important in defining what the elderly person eats and how he/she eats it and in extreme cases leading to anorexia of aging (47,48).

One of the reasons problems with taste and swallowing exist is the high prevalence of xerostomia (49–51). This condition is characterized by a diminished saliva flow and produces mouth dryness (11). This pathology is also one of the risk factors for caries progression (52,53) and periodontal disease (11,54) since saliva has many protective functions. Though it is associated with aging, studies have shown that salivary gland functioning is commonly found to be sound in healthy older populations (49,51). Thus, the etiology of xerostomia is probably of systemic or extrinsic foundation and it can be caused by medication such as psychotropic agents, antihistamines, and diuretics (50,51) often present in the prescription

regimens of this age group. In this thesis we are not evaluating xerostomia directly but it can be inferred through salivary flux measurement (55).

The close relationship between oral and systemic health means that it is important to follow closely the oral health status and promote and maintain oral health in order to have a better systemic health and to achieve individual wellbeing.

1.4. Self-perceived oral health assessment

The World Health Organization suggests that health is defined not only by the absence of disease but also by subjective well-being (health-related quality of life) (56). A widely used means of evaluation of oral health related quality of life is the Geriatric Oral Health Assessment Index (GOHAI) (57,58). This multidimensional index evaluates three main aspects: physical function, through chewing, speaking and swallowing patterns; psychosocial function, through the concern with oral health and satisfaction level regarding mouth esthetics; and pain or discomfort, evaluated by the analgesic intake (57). Through this questionnaire, it is possible to measure elderly self-perception of oral health (57). This index results in a final classification of self-perception classified as “high” (34 to 36 points), “moderate” (30 to 33 points) and “low” (< 30 points) (57). This score can be translated to the life quality related with oral health (57–59).

The information obtained from the application of instruments such as GOHAI is essential on the one hand for the accurate knowledge of the epidemiology of oral health in the Portuguese elderly, and on the other hand for the development of social actions targeting the prevention, diagnostics and interventions in this particular population. How the elderly perceive their oral health is a fundamental factor to guide the oral health professionals in the development of oral health policies and education programs which are effective and adapted to specific populations (57).

2. Saliva as a diagnostic fluid

Saliva is an oral fluid, product of the salivary glands, mostly composed by water (99% of its constitution), but includes electrolytes, proteins, enzymes and other non-glandular components like blood cells, food debris, microorganisms and microbial products (60,61). This fluid is very important due to its role in various processes, such as maintaining homeostasis and oral health, helping with food digestion and taste perception and defense against pathogenic microorganisms (62).

‘‘Salivary diagnosis’’ has been a part of strategic planning of the National Institute for Dental and Craniofacial Research (NIDCR) due to its role in providing scientific support to improve dental, oral, and craniofacial health. Saliva has been considered a diagnostic fluid (61–64) due to several reasons and is especially interesting for large epidemiological studies (65). When compared to blood collection, saliva is a safer and less invasive method and processing and collection costs are reduced (61,66). Although a great need still exists for convenient and accurate point-of-care devices that can serve as a non-invasive diagnostic, this issues will soon be overcome (64) and several prototypes exist in the market (67,68).

It is known that saliva mirrors local and systemic fluctuations (64,69), since it allows the study of different molecules, DNA, RNA, proteins, metabolites and the microbiome (70). Currently, saliva is used in fields of medicine, dentistry, pharmacotherapy, epidemiology (64), diagnosis of periodontal and other oral diseases, but studies are being conducted to extend its diagnostic capacities to systemic diseases, such as asthma, diabetes, and cancer (60,64). In the specific case of cancer, liquid biopsies from saliva present an interesting approach which, upon a wider validation, can substantially change the way we look at cancer diagnostics (64,70).

Although a saliva collection can be an easy method to assess elderly’s health, it can also be problematic since the elderly can suffer from xerostomia or hypo salivation (12,49,51). Nevertheless, with a stimulated saliva collection method (stimulating the salivary glands to produce more saliva) this issue can be overcome (71). Additionally, a study by Dhima and others (2013) (72) has found that adult patients prefer giving saliva when compared to other fluids such as urine or blood. Therefore, it can be stated that saliva has become increasingly popular as a diagnostic fluid both to the research community and to the patients/users.

2.1. SalivaPrint - total protein profile analysis

Proteins constitute 1% of saliva’s total, with 3397 proteins described as present in the oral cavity (73). For the protein analysis including determination the protein profile for each individual and a correlate it with their health condition,

capillary electrophoresis is a useful tool. The principle for this technique is based on the proteins' separation according to the molecular weight. The results are shown in a graphic with different peaks, each characterizing not one, but several proteins, all within the molecular weight range (61). The protein profile evaluates the presence or absence of a molecular weight range which can be associated with a biological state demonstrating physio-pathological changes (74). We envision the use of SalivaPrint in a twofold approach: i) on the one hand it provides a "quality control" strategy to ascertain the quantity and quality of the proteins present in the samples collected, guiding future research applications of the sample; and ii) it is possible that the individual characteristics of the saliva and the microbiome are reflected by this total protein profile and therefore SalivaPrint might be an useful resource for individual/patient stratification.

2.2. Microbiome

The human microbiome is estimated to be ten times the human body cells number (75). The microbial communities are regular residents of the skin, the oral cavity, vaginal and intestinal mucosa and are involved in several essential functions contributing for the host wellbeing (76). However, even the equilibrium between the host and the microbiome is altered, dysbioses occur which result in disease (77).

Technology evolved in the direction of culture independent molecular methods. This allows the detection of the great diversity of microorganisms (78). The human microbiome contributes with functional genes and metabolites which affect human physiology and are, therefore, considered an important factor for maintaining health. (78).

Inter-individual variation in the microbiome is specific, functionally relevant and personalized. One example of this is illustrated by the *Streptococcus* spp. of the oral cavity from different individuals (79). Through life (infancy to old age) the individual undergoes several physiological changes which include changes in the microbiome (80). The microbiome of an infant and the geriatric individuals is more similar between them than to the adult microbiome (78). For instance in oral microbiome, from infancy to old age the phyla ratio is transformed, only *Firmicutes*

phylum maintains the highest percentage (78). Also, *Bacteroidetes* phylum is present in higher number on infants than on elderly individuals (78). In oral microbiome as in the gut microbiome there is an increase in bacterial diversity with age (78,81). Ora and gut microbiomes will be addressed ahead.

2.2.1. Oral microbiome

The human species comprises a huge diversity of microbiomes that includes approximately 10,000 different microorganisms, distributed into 16 different phyla, four of which are of great relevance in oral health: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (77,82,83). Data from the NIH Human Microbiome Project showed that, among unrelated individuals, the oral microbiome has the ”larger core of commonly shared microbes” (84).

The oral cavity is a main entrance of microbes (78) and has distinct habitats for bacterial colonization which can be grouped into shedding and solid surfaces (77). The shedding surfaces correspond to the soft tissues of the oral mucosa and include the tongue, cheeks and gingiva. These surfaces with different degrees of shedding provide a “temporary” habitat for microbes and the biofilms formed in these surfaces are quite different from those formed in hard surfaces. The latter are mainly composed by the enamel and cement which are the dental hard surfaces exposed to colonization. These surfaces, although similar in chemical nature of the substrate, vary widely in the abiotic and biotic factors presented to the microbial colonization. For example, it is obvious that although the crown of a molar and the interdental surface of the incisors present the same substrate (acquired pellicle covered hydroxyapatite), the conditions of friction, exposure to saliva, pH and oxygen levels are expected to be quite different. The differences in abiotic factors modulate the primary colonization by oral bacteria. This colonization becomes the initial layers of the biofilm which, if left undisturbed, will mature into a complex ecosystem composed of different microbial species capable of finding the right adhesion substrates and other abiotic and biotic conditions (nutrients, oxygen levels, pH and microbial synergies) to thrive. It is this biofilm community in its different stages of maturity which can interact with the host and constitutes de oral microbiome. In fact, this interaction with the host is another reason why the oral

biofilms are different, not only in an individual but also at different times. The oral microbiome is considered as extremely dynamic and the fact that is establishes a “continuum with the external environment” contributes to this fact (85). Furthermore, the eating, speaking, and immune defense “functions” present in the oral cavity also interfere with the microbial colonization. Another factor influencing the microbial colonization and the establishment of equilibrium between the host and the microbiome is saliva. As explained in section 2.1 saliva with its multiple functions in the oral cavity can directly and indirectly influence microbial colonization: the presence of anti and pro microbial molecules and the salivary flow are but two of the main factors influencing which bacteria colonize which surfaces. In fact, it has been determined that many of the inter individual differences seen in the microbiome may result from differences in salivary flow and saliva composition (86).

A healthy individual owns an oral microbiome composed mostly by *Firmicutes* (genus *Streptococcus*, family *Veillonellaceae*, genus *Granulicatella*), which constitutes 36% of the total, 25% *Actinobacteria* (genus *Corynebacterium*, *Rothia, Actinomyces*), 22% *Proteobacteria* (genus *Neisseria*, *Haemophilus*), 11% *Bacteroidetes* (genus *Prevotella*, *Capnocytophaga*, *Porphyromonas*) and 5% *Fusobacteria* (genus *Fusobacterium*) (77,87). It is striking that in spite of the intra and interindividual differences in the microbiome there seem to be identical bacterial sequences in the oral cavity of unrelated healthy individuals (77).

Oral microorganisms interact with host factors in the oral cavity (80) and an “oral microbial homeostasis”, the ability of the ecosystem to keep microbial stability in health is achieved (80). However, when the symbiotic balance between the host and the microbiota is lost, these microbiota can be involved in disease (77). Understanding, the microbiome changes in early stages of oral diseases, like dental caries and periodontitis, can be a of great value for the diagnosis and treatment before clinical signs appear (77).

Caries is an oral pathology in which microbiome components are the major etiological causes. However, caries active and caries-free individuals share approximately 50% of the supragingival microbiome (88). In caries active individuals 10 genera were present in high abundance including *Streptococcus* spp, *Veillonella* spp and *Actinomyces* spp. *S. mitis* (25,5%) and *S. sanguinis*, (9,1%), surprisingly *Streptococcus mutans* (1,2%) was a comparatively minor

constituent. The great majority of represented phenotypes were similar in both caries-free and caries-active children but decreased in caries-active samples. Phylotypes overrepresented in caries-active subjects included *S. sanguinis*, *S. mutans*, *S. sobrinus*, *S. mitis*, *S. intermedius*, *S. gordonii*, *S. parasanguinis*, *S. constellatus*, *S. cristatus*, *S. oralis*, *S. equi*, *S. dentirousetti* and *S. peroris*. In spite of *S. mutans* displaying the greatest differential abundance of the observed phylotypes, the fact that there is a wide spectrum of other overrepresented bacteria (of the *Streptococcus* genus) suggests that a *S. mutans* etiology is ambiguous in dental caries. This can be related to the fact that it seems that *S. mutans* is only associated with caries initiation (white spots) but not with caries progression. In this respect *S. mutans* appears to have the characteristics of a keystone pathogen or of a pathobiont driven by a changing dietary environment (89). In fact, the major factor involved in caries is pH and several species of the hard surface biofilm are both acidogenic and aciduric and seem to act synergistically to promote demineralization of the enamel.

The lack of a singular pathogen related to periodontal disease is also evident. In the case of this disease the role of the host is more relevant and when the gingival immune response fails, periodontal tissue pathology results. The impaired immune response results in the release of matrix metalloproteinases from neutrophils and T cells and mediate alveolar bone loss (89). In healthy gingival sulci (less than 4 mm deep), the phylum Proteobacteria, particularly the gammaproteobacteriae of genus *Acinetobacter*, *Haemophilus* and *Moraxella*, were most prevalent. Within the phylum *Firmicutes*, the class *Bacilli* comprising genus *Streptococcus*, *Granulicatella* and *Gemella* were also health-associated. These genera can be considered symbionts, which also return to periodontal pockets in high proportion after periodontal treatments (90). Conversely, bacteria such as *P.gingivalis*, *Tannerella forsythia* and *Treponema denticola* have historically been associated to deep periodontal pockets (91). These cultivable bacteria belong to the “red complex” proposed by Socransky and colleagues, but new sequencing technologies have facilitated novel associations between periodontitis and previously uncultivable or underappreciated species, including the Gram-positive *Filifactor alocis* (92) and *Peptostreptococcus stomatis*, and species from the genera *Prevotella*, *Synergistes* (93), *Megasphaera*, *Selenomonas*, and

Desulfobulbus (94). Several of these organisms correlate with disease as strongly as the classical red complex bacteria (89).

Understanding the microbiome fluctuations at early stages of oral diseases can be useful for early diagnosis and intervention (63,77). Thus, the comprehension of elderly person microbiome is an important tool for patient evaluation.

2.2.2. Gut microbiome /Oral microbiota comparison

The gut microbiome is well studied and there is more evidence regarding the interactions with the host for the gut than for the mouth. The gut microbiome is composed by the same main phyla as the oral microbiome (95) and the microbes are involved in many different processes in the human body, such as digestion, metabolite production, disease processes, usually analyzed through feces samples (66). The oral microbiome is associated with both oral and systemic diseases, probably since the oral cavity is the microbe's main way of entrance on our organism (63). Additionally, the human microbiome has great variety depending on many factors—age, geographical location, and host's genetics (78,83). Studies were done to define the characteristics of a healthy microbiome (77) and to determine the *Firmicutes*/*Bacteroidetes* ratio associated with health (96) since the composition of the human gut microbiome impacts host metabolism. Furthermore, Greenhalgh *et al*. (2016) have shown that in some cases a high ratio indicated metabolic diseases, but that some of the subjects were healthy showing the same ratio (78).

Firmicutes and *Bacteroidetes* phyla are present in the gastrointestinal tract in higher quantity (78). In opposition to the oral microbiome, in the gut, the main phylum is *Bacteroidetes*, followed by *Firmicutes* (78). For both microbiomes, it is true that the balance is changed through aging (78). Also, microbial diversity decreases with disease status generating a dysbiotic microbiome (63,95).

Our hypothesis is that, like the gut microbiome, the oral microbiome also plays an important role in health and disease conditions. It is possible, with a saliva collection, to perform an estimation of the total bacterial load and a *Firmicutes*/*Bacteroidetes* ratio calculation. Through culture-independent molecular

methods, which includes the non-cultured species, we could provide earlier diagnosis methods and consequently provide more accurate treatment options (77,82).

Goals

This work aims at establishing a multidimensional strategy towards the characterization of the oral health in a senior population through the:

a. Evaluation of lifestyle habits (diet type, place of residence and smoking habits), the general health (diabetes, hypertension, cardiovascular diseases), and oral health self-perception,
b. Characterization oral health status,
c. Study saliva's parameters (salivary flux, pH and protein concentration and total protein profile analysis)
d. Analysis of the oral microbiome through the total bacterial load and the *Firmicutes/Bacteroidetes* ratio.

Material and Methods

Material and Methods

This observational and cross-sectional study uses 3 main data collection strategies. Oral health was evaluated through clinical observations; demographic and self-reported oral and systemic health was evaluated through questionnaires; and a saliva sample was collected for molecular data analysis (Figure 1).

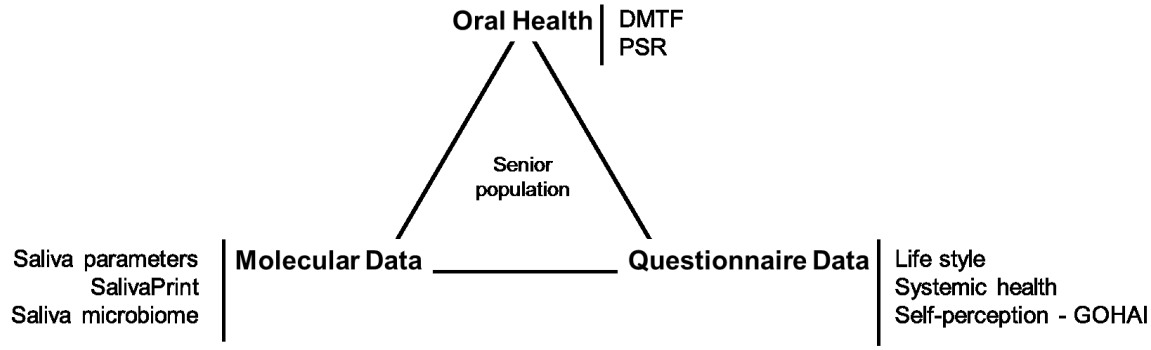


Figure 1. Study design: three main data collection strategies—oral health, questionnaire data, and molecular data analysis.

1. Characterization of the population

1.1. Population in study

A study group of 461 individuals from the population participating in the "Atividade Senior" program was studied. This program includes a series of physical activities designed for the capacities and needs of a senior population (http://www.cm-viseu.pt/index.php/diretorio/desporto/actividades-desportivas-municipais/actividade-senior). To all the participants in this program, the study was presented and an Informed Consent form was signed by all willing to participate.

1.2. Questionnaire applied

The sociodemographic, systemic health and diet type information was gathered through an extensive questionnaire (Annex 1).

The diet type questionnaire used was validated for the Portuguese population by Afonso, Moreira, & Oliveira, 2014 (97). Diet type was evaluated by the final score of the questionnaire (maximum 12), separating in a Not Mediterranean diet (score<10) or a Mediterranean diet (score ≥10) (97).

The Geriatric Oral Health Assessment Index (GOHAI) applied was validated by Carvalho, C. *et al*. (2013) (57). The GOHAI evaluated the oral health issues in three dimensions: physical, psychosocial, and pain and discomfort , classifying the subject in: high self- perception (score 34-36), moderate self- perception (score 30-33) and low self- perception (score <30) (57).

1.3. Oral health evaluation

The oral health status was evaluated by the examination methods and criteria recommended by WHO (4). The oral health status was assessed by using a mouth mirror illuminated by an extraoral light-emitting diode. A team of trained dental students performed all the oral examinations. Firstly, the DMFT index was measured and the data registered included teeth with caries (decayed teeth were detected at the cavitation level), lost teeth and filled cavities (Table I) (4). Afterwards, the DMFT index was calculated, consisting of the sample's total average number of decayed, missed and filled teeth, including the third molars (4). Additionally, the teeth visual and tactile inspection was performed. No radiographs were taken.

Table I. Oral health status code (4).

Code	Criteria
0	Healthy
1	Carious lesion
2	Filled cavities with carie
3	Filled cavities without carie
4	Lost tooth by carie
5	Lost tooth (by other reason)
6	Sealant
7	Prosthesis or implant
8	Not erupted
T	Trauma
9	Not registered

Finally, the severity and degree of periodontal diseases (gingivitis, periodontitis) for each subject was assessed, according to a WHO-recommendation (4), by the Periodontal Screening and Recording Index (PSR) which is similar to the Community Periodontal Index of Treatment Needs. For the clinical evaluation a Click-Probe® was used (98,99). The following parameters were evaluated: bleeding on probing, dental calculus, and gingival sulcus. Periodontal disease were classified into five degrees according to their severity ranging from 0 (healthy) to 4 (most severe form of periodontitis) (Table II) (15,99). The periodontal state was divided in four severity levels: gingivitis (class 1), slight (class 2), moderate (class 3) and severe periodontal disease (class 4) (Table III) (98,100).

Table II. Periodontal Screening and Recording score (15,99).

Score	Criteria
0	Periodontal health
1	Bleeding on probe (BOP)
2	Dental calculus detected during probing, iatrogenic margin and/or BOP
3	Periodontal pocket 3,5-5,5 mm
4	Periodontal pocket 6 mm or more
*	Periodontal abnormalities present (furcation involvement, tooth mobility, gingival recession, muco-gingival problems)

Table III. Clinical degrees of severity and diagnostic parameters (90).

Clinical Degrees of Severity		Probing Depths	Clinical Attachment Loss	Furcation Invasion	Tooth Mobility
Class	Form				
1	Gingivitis	1-3 mm	-	-	-
2	Slight Periodontitis	4-5 mm	1-2 mm	-	-
3	Moderate Periodontitis	5-7 mm	3-4 mm	~F1	+
4	Severe Periodontitis	>7 mm	≥5 mm	F2, F3	++

1.4. Saliva sampling

Saliva collection can be done through different methods, for this study we followed two different approaches unstimulated and stimulated saliva collection.

Unstimulated whole saliva (UWS) was collected and processed according to Rosa N. and colleagues (2016) (42) standard operating procedure (SOP). Briefly, subjects are asked to refrain from eating, drinking or have oral hygiene procedures 1 hour prior to saliva collection. Before collection, subjects are asked to rinse the mouth with clean water for 30 seconds to remove desquamated epithelial cells, microorganisms and food and drink remnants. After the mouth rinse, subjects are asked to wait for a minute before collection. Two cotton rolls are placed in the oral vestibule for 2 minutes. To recover the saliva sample (1–2 mL), cotton rolls are collected in a plastic tube and centrifuged at 10000 × g for 10 minutes. Supernatant is collected, aliquoted and stored at –80ºC until analysis. In order to have samples of different stages of physical activity program, samples were collected at 3 time points: in February, in June and in December 2016. The samples collected were stored at Biobanco-SalivaTec, Catholic University of Portugal, Viseu, Portugal. Additionally, a 10 mL mouthwash of water was collected for further DNA isolation.

Stimulated whole saliva was collected according to Mussi *et al*. (2016) (43) during exactly 5 minutes. This protocol was implemented in our lab in February 2017 and will be continued through future collections that are not included in this thesis.

1.4.1. Saliva parameters

1.4.1.1. Physicochemical parameters

The salivary flow rate was measured by calculating the microliters of saliva produced per minute. The pH was measured in a pH meter (Hanna Instruments) and protein concentration (µg/mL) was measured by spectrophotometry (using Protein UV protocol with NanoVueTM Plus, GE Healthcare, PT).

1.4.1.2. SalivaPrint - Total protein profile analysis

A total protein profile was obtained by capillary electrophoresis (SalivaPrint) for each sample. SalivaPrint was produced using the Experion ™ Automated Electrophoresis System (Biorad) in standard protein chips (Experion ™ Pro260 Analysis Kit). The samples were analyzed according to the technical specifications provided by the manufacturer. Briefly, 4 µL of sample is mixed with 2 µL of a reducing buffer and boiled for 10 minutes at 95-100 ° C. Subsequently, 84 µL of ultrapure water were added to each sample. Each chip was impregnated with gel and staining gel in a priming station and charged with 6 µL of each sample prepared as described above. The Pro260 molecular weight marker included in the kit was used as standard. The calibration curve used in all analyzes were done using an internal standard provided by the manufacturer. This method has a resolution of 2.5-2000 ng/mL of protein and manages to separate proteins from 10 - 260 kDa. The results were analyzed using Experion™ Software, version 3.20. Proteins found in the different ranges were identified by comparison with the results from Rosa et al 2016 (74) and their association with disease was obtained from OralCard database (73).

1.4.2. Saliva microbiome characterization

1.4.2.1. Extraction of DNA from pure cultures and saliva

DNA was obtained from the 10 mL mouthwash samples using the InstaGene Matrix® (Bio-Rad, Lisbon, PT) and following manufacturer's instructions. Briefly samples were thawed on ice and centrifuged at 12000 rpm for 1 minute and the supernatant removed. The pellet was re-suspended in 200 µL of the InstaGene Matrix and incubated first for 30 minutes at 56ºC and then boiled for 8 minutes. Following centrifugation, a final volume of 150 µL of supernatant was obtained and the DNA concentration and quality (280/260 nm absorbance) was determined by spectrophotometry (NanoVueTM Plus, GE Healthcare, PT).

1.4.2.2. qPCR reaction

In order to quantify the bacterial load (all bacteria) the *Bacteroidetes* and *Firmicutes* phyla the SsoFast™ EvaGreen® Supermix (Bio-Rad®) was used in conjugation with a set of primers for each group (Table IV) (38). The amplification and detection of DNA by real-time PCR was performed with the CFX Connect™ Real-Time System (Bio-Rad®) using optical grade 96-well plates. Samples were used for bacterial quantification by real-time PCR. The PCR reaction was performed in a total volume of 10 µL containing 100 nmol/L of each of the forward and reverse primers and 1 ng of sample DNA. The PCR reaction conditions for DNA amplification were 98°C for 2 min, 98°C for 5 seconds and 60ºC for 5 seconds, the last two steps were repeated during 40 cycles. A melting curve analysis was done after amplification.

Table IV. Sequence of oligonucleotide primers, product size (bp) and annealing temperature for each assay (101).

Assay	**Primer name and sequence (5´-3')**		**Product size (bp)**	**Annealing temperature (ºC)**
Bacteroidetes	Bact934F	GGARCATGTGGTTTAATTCGATGAT	126	60
	Bact1060R	AGCTGACGACAACCATGCAG		
Firmicutes	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	126	60
	Firm1060R	AGCTGACGACAACCATGCAC		
All bacteria	Eub338F	ACTCCTACGGGAGGCAGCAG	200	60
	Eub518R	ATTACCGCGGCTGCTGG		
Bacteria 16S rRNA	Eub338F	ACTCCTACGGGAGGCAGCAG	1049	56
	Eub1369R	CCGRGAACGTATTCACCG		

1.4.2.3. Standard curve

Quantitative analysis implies that a standard curve is built with known concentrations for the fragments to be amplified in the PCR reaction. For the *Firmicutes* a standard curve was obtained from *Staphylococcus aureus* (specie confirmed by sequencing analysis). DNA obtained from a pure culture of *Staph. aureus* was amplified with Firm934F and Firm1060R set of primers. The amplified

product purified by NZYGelpure (nzytech Lisbon, PT) was cloned into the pNZY28 vector (nzytech, Lisbon, PT) followed by transformation into *E. coli* NZYStar Competent cells (nzytech, Lisbon, PT). The plasmid containing the fragment of interest was used as standard for *Firmicutes* phylum.

A similar procedure was performed for the *Bacteroidetes* phylum with *Porphyromonas gingivalis* ATCC 20709T using Bact934F and Bact1060R set of primers.

Six nonzero standard serial dilutions were analyzed in triplicate in each assay. The copy numbers of the target group for each reaction were calculated from the standard curves by dividing the quantification obtained by the initial DNA concentration.

2. Statistical analysis

The population sociodemographic and health data was analyzed by t-test and Ordinary one-way ANOVA (Graph Pad PRISM version 7.0, USA).

Total protein profile analysis was completed by a bioinformatics approach according to Rosa N. and colleagues 2016 (74).

Statistically significant differences on the microbiome data were identified by Kruskal-Wallis test; Ordinary one-way ANOVA, t-test (Graph Pad PRISM version 7.0, USA).

Results

Results

1. Characterization of the population

1.1. Sociodemographic characterization

During 2016 and 2017, 256 and 310 individuals, respectively, participated in this study. One hundred and five of these individuals took part, at least, once in each year. The majority of subjects are female, and the median age is 69 years of age (Table V). The population evaluated represents a sample from different Freguesias of Viseu and the collection took place in 12 different locations (Figure 2). At each site volunteers filled a questionnaire, provided a saliva sample and were observed regarding their oral health.

Table V - Participants characterization regarding sociodemographic and lifestyle aspects.

Characteristics	**% individuals**
Gender (n=337)	
Female	71,5
Male	28,49
Age by gender (n=337)	**Min. – Max**. (Mean ± SD)
Female	47 - 92 (69,44 ± 7,91)
Male	54 - 93 (71,09 ± 7,80)
Diet type (n=91)	
Mediterranean	35
Not Mediterranean	65
Place of residence (n=337)	
Rural	66,66
Urban	33,33
Smoking habits (n=337)	
Smoker	1,48
Non-smoker	78,64
Ex-smoker	19,88

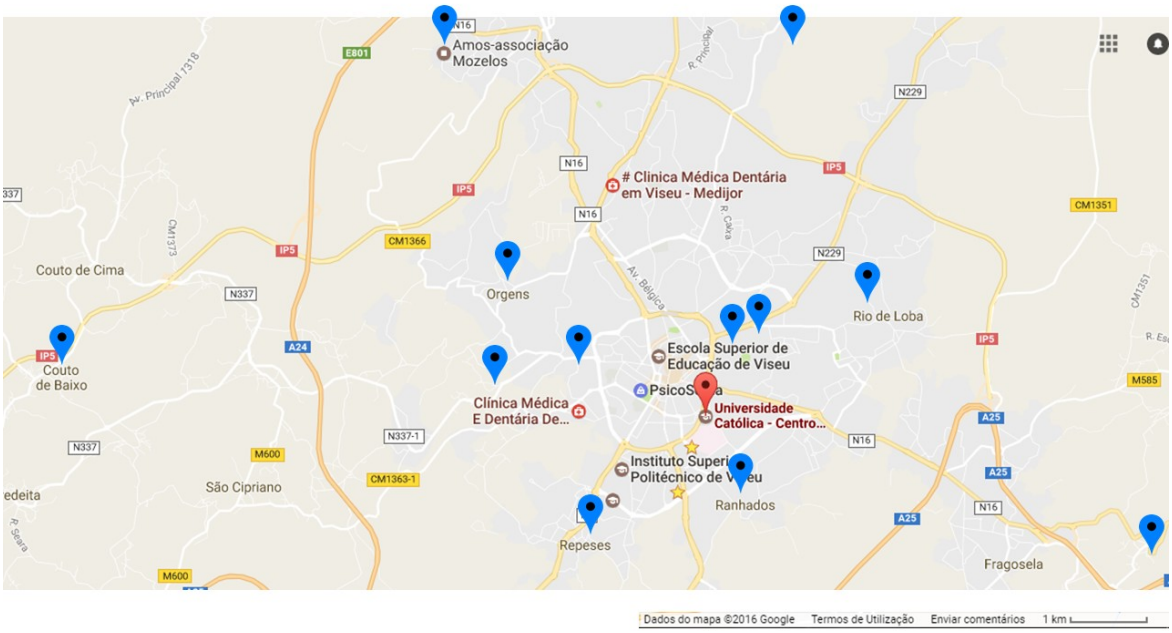


Figure 2. Data and saliva collection locations spanning 12 locations in the Viseu district.

Regarding diet, the mean score by age group doesn't differ (p=0,1211 with Ordinary one-way ANOVA) and all means are below 10 (<10 = Not Mediterranean diet). Female diet type mean score is slightly lower (8,613) than male (9,034), although there aren't statistically differences between them (p=0,2021 with Mann Whitney test). This indicates that the population observed doesn´t follow a Mediterranean diet.

1.2. Systemic health status of the population

Based on a self-reported questionnaire we were able to characterize the individuals regarding systemic health (

Table VI). The most distinctive feature is that there is a high percentage of hypertension cases (52%) and that most participants report not having any cardiovascular complications (92,58%) nor diabetes (85,63%).

Table VI - Individual's characterization regarding systemic health.

Characteristics	**% individuals**
Diabetes (n=334)	
Diabetic	14,37
Type 1	0,89
Type 2	13,13
Not Diabetic	85,63
Hypertension (n=335)	
Hypertensive	51,94
Not Hypertensive	48,06
Cardiovascular (n=337)	
With	7,42
Without	92,58

The results of the analysis of oral health self-perception are presented in Figure 3 and it can be observed that most participants (49%) consider having a satisfactory oral health, reporting no pain. Only 19% of them refer as to having poor oral health and the most common aspect indicated is "had a problem in the mouth which worried me". The mean GOHAI score for the population was 32,68±3,3 corresponding to a high oral health self-perception.

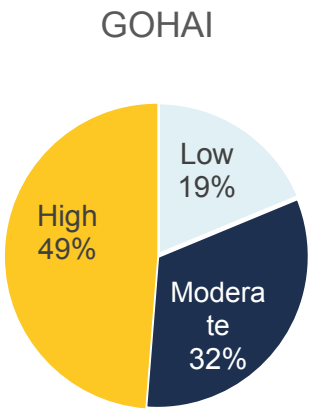
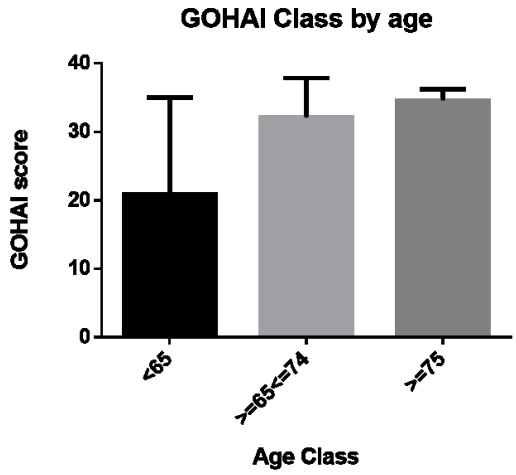


Figure 3. Distribution of participants by degree of oral health self-perception. High (score 34-36), moderate (score 30-33) and low self- perception (score <30) (n=216).

When associating GOHAI classes to age, statistically significant differences were found between the <65 group and the other two age classes, [≥65-74] and ≥75 (Figure 4). People under 65 years of age find that their oral health is worse than elderly people do.



Kruskal-Wallis test	P value <0.0001	
Dunn's multiple comparisons test	Summary	Adjusted P value
<65 vs. >=65<=74	***	0.0005
<65 vs. >=75	****	<0.0001
>=65<74 vs. >=75	ns	0.2664

Figure 4. Kruskal-Wallis test for GOHAI by age class—high (score 34-36), moderate (score 30-33) and low self-perception (score <30).

1.3. Oral health evaluation

1.3.1. Caries index (DMFT)

A subset of the initial population (n=109) was evaluated regarding their oral health status (Table VII).

Table VII - Oral health status of the senior population (n=109) (**p<0,01; ***p<0,001).

	Female	**Male**	**Total**
	mean ± SD	mean ± SD	mean ± SD
Decayed teeth	3,05 ± 2,76	3,33 ± 2,66	3,15 ± 2,73
Missing teeth	16,34 ± 9,35**	11,42 ± 7,84**	14,72 ± 9,18
Filled teeth	3,07 ± 3,77	2,78 ± 3,16	2,97 ± 3,58
DMFT index	22,44 ± 7,23	17,53 ± 6,65	20,82 ± 7,41***

Although decayed and filled teeth had a low prevalence among the population, the missing teeth were very prevalent. Statistically significant differences between the DMFT index by gender (p=0,0052) (Figure 5) were found and males show a lower value for DMFT index (17,53 ± 6,65). When each component of DMFT index is analyzed separately, only missing teeth show statistical differences between genders (p=0,0010) and females have more missing teeth than males.

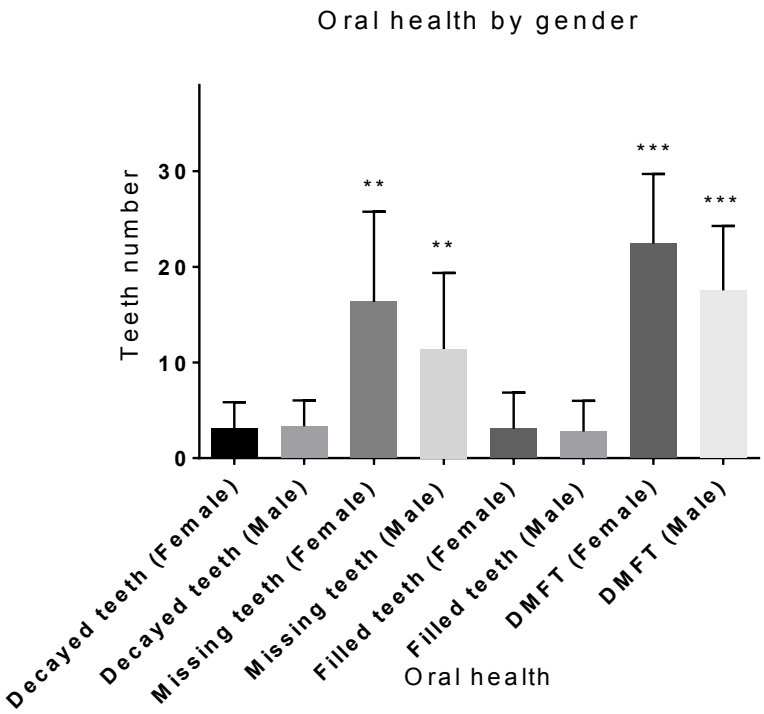


Figure 5. DMFT index (decayed, missing and filled teeth) and respective parameters by gender (**p<0,01; ***p<0,001)

Considering the whole population, missing teeth represent more than 50% of the total, 10% are decayed and the remaining are either filled (7%) or healthy (28%) (Figure 6).

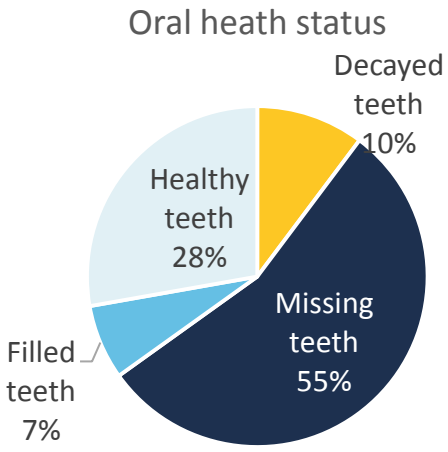


Figure 6. Teeth in different stages (decayed, missing, filled, healthy teeth) considering all participants of the group of study (n=109).

1.3.2. Periodontal index (PSR)

The results for the periodontal status of the population are presented in Figure 7. Chronic periodontal disease (PD) is present in 78% of the population. It is noteworthy that slight periodontitis was the most prevalent condition (42%), followed by the moderate periodontitis (23%) and only 3% of the studied individuals exhibited periodontal health (Figure 7). Four of them, that had moderate periodontitis, also had slight periodontitis in another sextant. Furthermore, individuals with severe periodontitis had slight (n=2) and moderate periodontitis (n=4) simultaneously. Despite not being subject of periodontal analysis, the edentulous subjects are included in the counts in order to have a real percentage of oral health for the whole group. Edentulous individuals probably represent previous situations of periodontal disease.

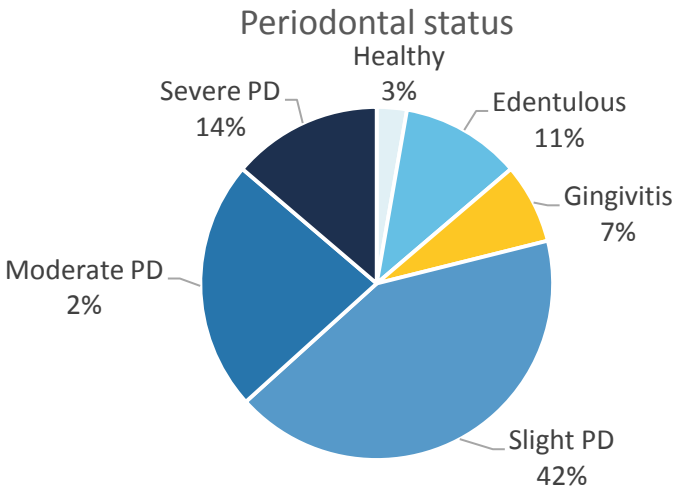
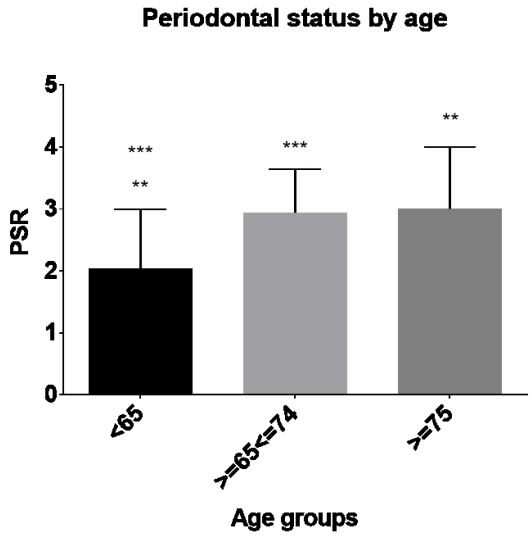


Figure 7. Percentage of each PSR level (healthy, gingivitis, slight, moderate or severe periodontitis) in the study group (n=109). Edentulous included as explained in text.

Periodontal status was compared in different age classes (Figure 8). Results show that there are statistically significant differences between individuals under the age of 65, that have better periodontal status than the other two age classes, [≥65-74] ($p < 0,0024$) and ≥75 ($p < 0,0004$).



ANOVA test	P value <0.0001	
Tukey's multiple comparisons test	Summary	Adjusted P value
<65 vs. >=65<=74	***	0,0024
<65 vs. >=75	****	0,0004
>=65<74 vs. >=75	ns	

Figure 8. Periodontal status (PSR) by age groups (n=97).

1.3.3. Correlation between oral health indices

Correlations between the different parameters analyzed in oral health, both clinically determined and self-perceived, were calculated and are presented in Figures 8-10. The DMFT index is not correlated with the PSR index (Figure 9), which is also true for the correlations between each oral health index and the self-perception of health Figure 10 and Figure 11.

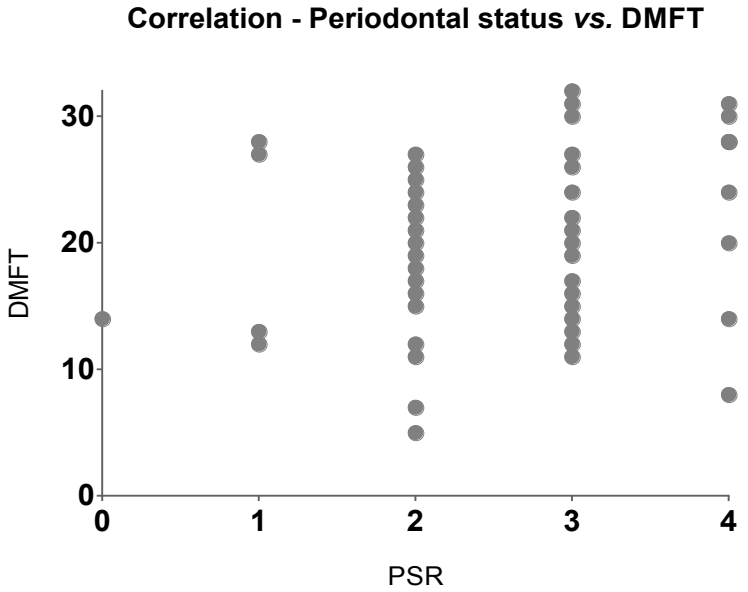


Figure 9. Correlation between the periodontal class (0 – healthy, 1 - gingivitis, 2 - slight periodontitis, 3 – moderate periodontitis, 4 - severe periodontitis) and DMFT index (0 - minimum, 32 - maximum) Spearman r = 0,2124.

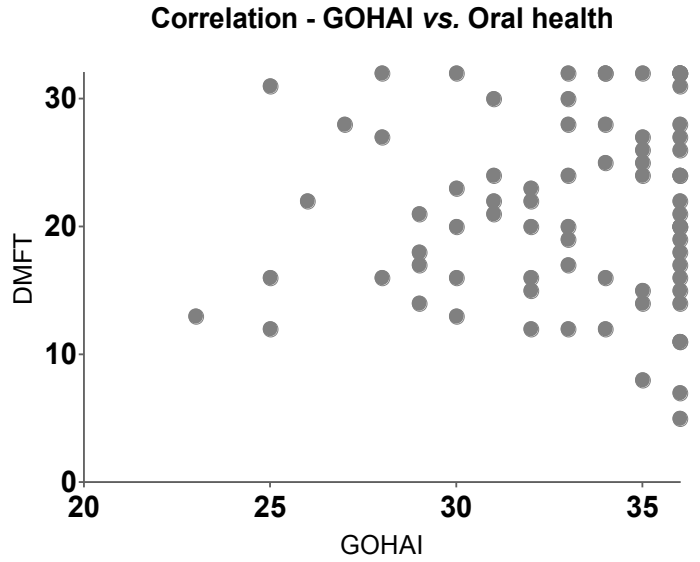


Figure 10. Correlation between GOHAI—high (score 34-36), moderate (score 30-33) and low self-perception (score <30)—and oral health (DMFT index: 0 - minimum, 32 - maximum) Spearman r = 0,07005.

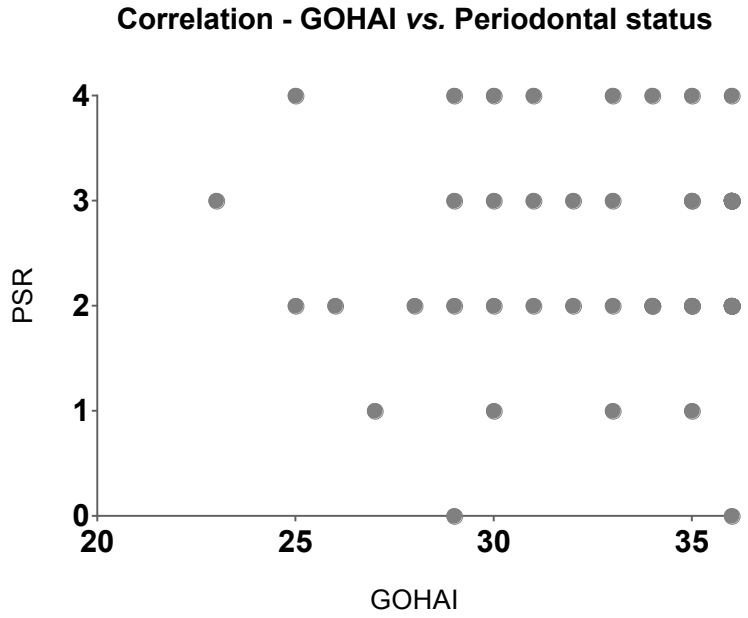


Figure 11. Correlation between GOHAI—high (score 34-36), moderate (score 30-33) and low self- perception (score <30)—and PSR (0 – healthy, 1 - gingivitis, 2 - slight periodontitis, 3 – moderate periodontitis, 4 – severe periodontitis) Spearman r = 0,06765.

Regarding the diet score relation with DMFT the means are slightly lower in Mediterranean diet type (20,69) when compared to Not Mediterranean (21,07). Although, unpaired t test showed no statistical differences (p=0,8131).

Concerning the diet score relation with PSR the means are slightly different between Mediterranean (2,310) and Not Mediterranean (2,346). Although, unpaired t test showed no statistical differences (p=0,8718).

Self-perception of oral health is correlated with the diet type, since both means fit in moderate self-perception, with no statistical differences (0,2421 with Mann-Whitney test).

When looking at the diet type and systemic conditions there were no statistically significant differences. Nevertheless, the diabetic individuals seem to have more frequently a Mediterranean diet than non-diabetic.

1.4. Saliva parameters

1.4.1. Physicochemical parameters

Saliva samples measurements were done for: salivary flow rate, pH and protein concentration (Table VIII). When comparing the differences in the flux by genders there were statistically significant differences ($p < 0,0001$) for the stimulated method and, males showed a higher flux than females (Figure 12).

Table VIII. Saliva parameters (Mean ± Standard Deviation).

Saliva collection method	Collection time points	Flux (µL/min)	pH	Total Protein Concentration (mg/ml)
Unstimulated	1	422,18 ± 328,77	7,27 ± 0,56	4742,15 ± 1696,41
	2	337,78 ± 265,53	7,14 ± 0,55	4425,50 ± 2593,16
	3	293,02 ± 232,58	6,90 ± 0,59	2930,44 ± 2460,97
Stimulated	1	1414,81 ± 665,72	7,63 ± 0,41	4391,59 ± 2590,62

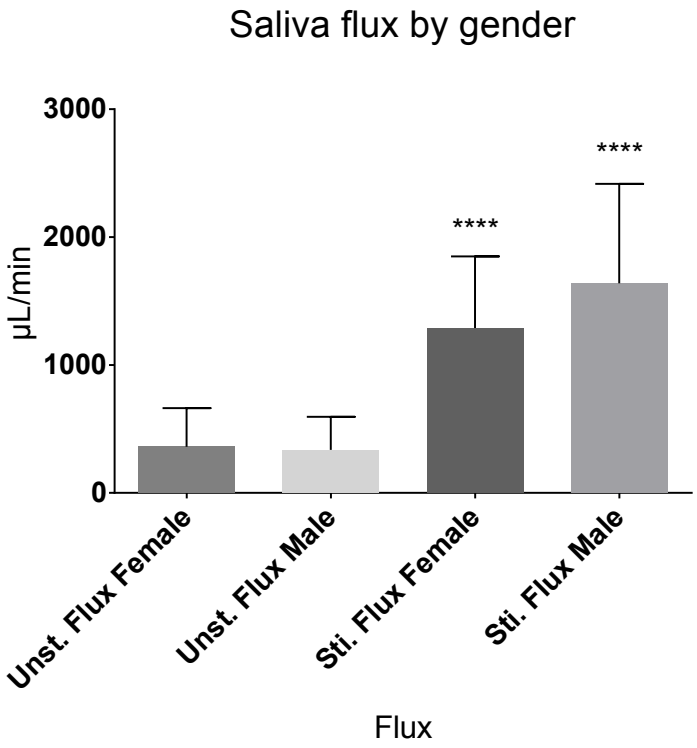


Figure 12. Two-way ANOVA (α = 0,05): flux for unstimulated (Unst.) and stimulated (Sti.) saliva collection by gender (****$p < 0,0001$).

1.4.2. SalivaPrint - Total protein profile analysis

With the capillary electrophoresis, the total protein profiles for each individual were obtained. The individuals used in this section had data in all the dimensions evaluated in this study, (diet score, oral health status, two sampling times), (n=40). Total protein profiles of the saliva samples were grouped according to the periodontal state of individuals. The most typical profiles were selected: 3 healthy, 4 edentulous, 4 gingivitis and 4 periodontitis (Figure 13). This strategy allows the observation of an interindividual variation in the SalivaPrint (arrows in Figure 13). There are also differences in the total protein profile between the oral health conditions (Figure 13 and 14). The observed changes, are mainly in the concentration of the proteins and the profiles superimpose very well, except for the peak in Figure 13d.

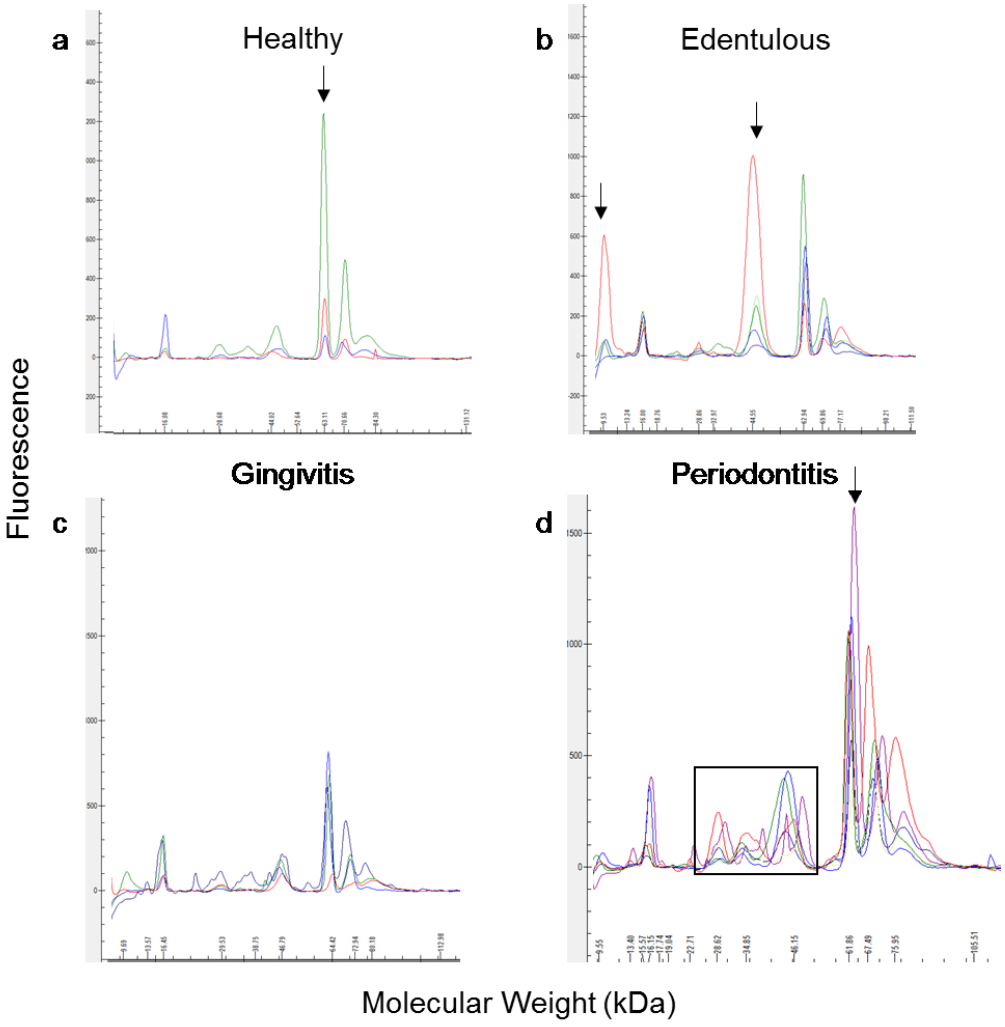


Figure 13. Total protein profiles for: a – healthy, b - edentulous, c - gingivitis, and d - periodontitis individuals (arrow=distinct peak; box = variable range) (n=40).

Figure 14 shows the representative profile for each oral condition and it can be seen that for the edentulous patients there are two molecular weight ranges in which the protein concentrations are higher than in other groups. Additionally, participants with periodontal disease also show a molecular weight range where the profile is quite different from all other conditions (arrows in Figure 14). Furthermore, there is a molecular weight range where changes related to the oral health status (box in figure 14).

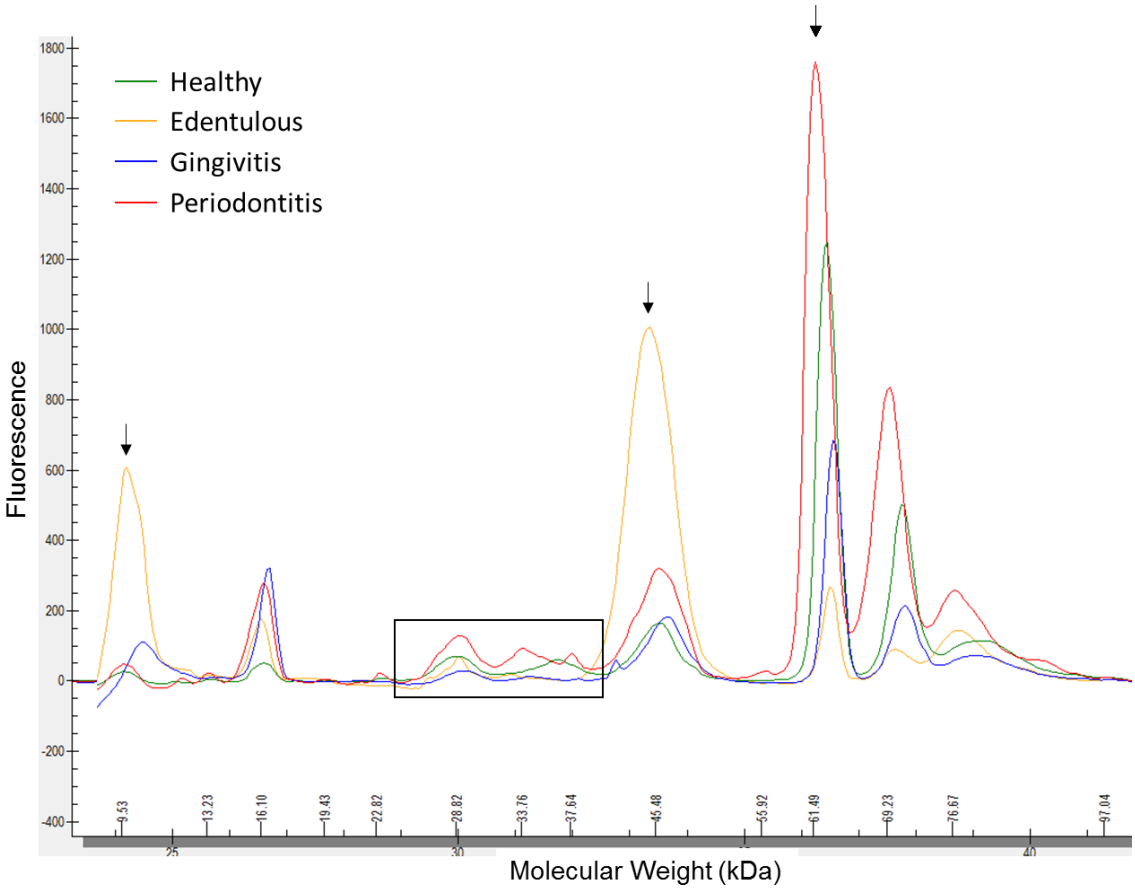


Figure 14. Typical protein profile *per* periodontal condition—healthy, edentulous, gingivitis, and periodontitis (arrow = higher peak; box = variable range).

In order to unveil the molecular weights range that have more discriminant capacity, we performed bioinformatic data analysis and compared the profiles obtained from our elderly population with a known healthy population from the SalivaTec biobank. The goal was to verify which molecular weight ranges better discriminate individuals with periodontal disease and propose proteins related to periodontal diseases development based on the molecular weight range. It was possible to learn that molecular weights in the range 28-29, 42-43, and 77-78 kDa

are related to the health *vs.* periodontal disease classification (Figure 15). Results are shown in Table IX. Some of the molecular weight ranges correspond to proteins associated with periodontitis and peri-implantitis conditions.

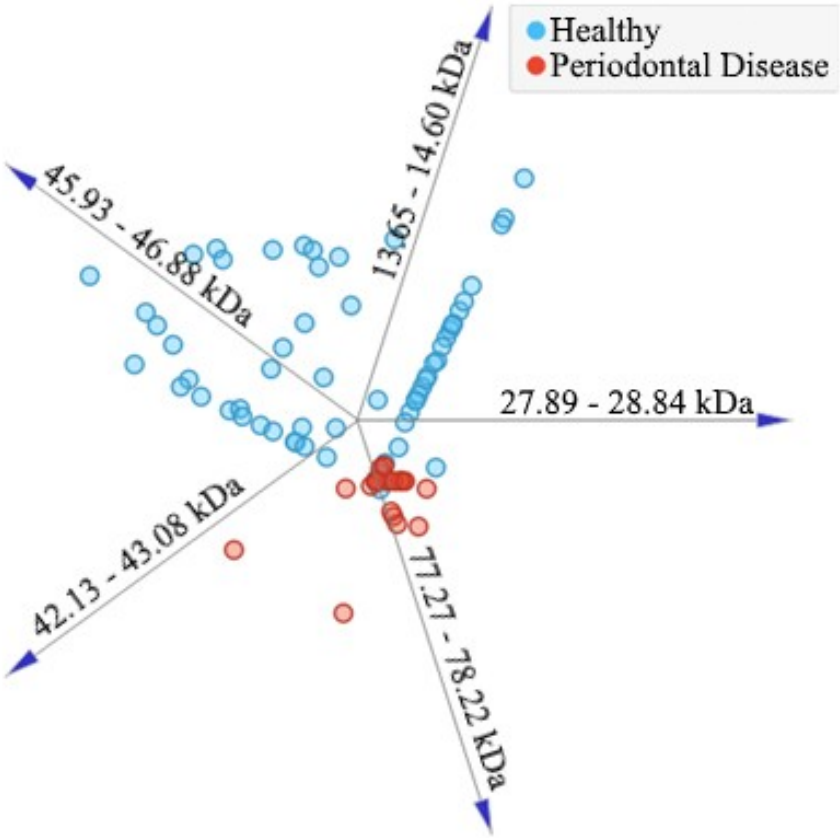


Figure 15. Molecular weight ranges (kDa) contributing to discrimination of healthy *vs*. periodontally diseased individuals.

The proteins associated with the molecular weight represented in Figure 13 are shown in Table IX. These associations were established with proteins previously identified by mass spectrophotometry (74). Some of the molecular weight ranges correspond to proteins associated with periodontitis and peri-implantitis conditions (73).

Table IX. Protein names, molecular weight (kDa) and related health condition (73,74).

UniProt code	Protein names	Molecular Weight (kDa)	Condition	Molecular Weight Range (kDa)
P31947	14-3-3 protein sigma	28	Periodontitis/ Healthy	28 - 29
P06870	Kallikrein-1	29	Healthy	
P30740	Leukocyte elastase inhibitor	43	Healthy	42 - 43
Q08188	Protein-glutamine gamma-glutamyltransferase E	77	Healthy	77 - 78
P02788	Lactotransferrin	78	Periodontitis/ Healthy	

1.4.3. Saliva microbiome characterization

In order to characterize the subject's microbiome, an estimation of the total bacterial load, as well as the quantification of *Firmicutes* and *Bacteroidetes* phyla by qPCR was performed.

A summary of the microbiome results is presented in

Table X. The sociodemographic and health data is not correlated to 16S quantity and *Firmicutes*/*Bacteroidetes* (F/B) ratio. Nevertheless, some trends can be observed. Concerning the gender, female seem to have a lower total bacteria load (mean=14502) and a lower F/B ratio (F/B=12,1) comparing to males. Also, the under 65 age group mean of the total bacteria load (mean=16943) seems higher than the other age groups and showed a lower F/B ration (F/B=10,87). Regarding individuals with a Mediterranean diet type they presented a higher mean total bacteria load (mean=16814) and a lower F/B ratio (F/B=6,940) than individuals with a non-Mediterranean diet (F/B=16,95).

Table X. Quantification means and p-values for 16S all bacteria and Firmicutes/Bacteroidetes ratio (number of sequence/ng of DNA) for sociodemographic data ([1]t-test, [2]Kruskal-Wallis test).

	16S all bacteria	16S all bacteria P-value	Firmicutes/ Bacteroidetes ratio	Firmicutes/ Bacteroidetes ratio P-value
Gender[1]				
Female	14502	0,7769	12,1	0,5784
Male	15540		14,53	
Age ranges[2]				
<65	16943		10,87	
>65≤74	12887	0,4632	14,67	0,5926
≥75	11777		12,05	
Diet type[1]				
Mediterranean	16814	0,2764	6,940	0,3012
Not Mediterranean	13121		16,95	
GOHAI- Self-perception[2]				
Low	12965		9,949	
Moderate	17053	0,7905	7,782	0,2693
High	12587		18,91	

Table XI presents the microbiome study results considering oral and systemic health data. The group with the lower DMFT index (0-12) showed a higher total bacteria load (mean=22161) than the others, and a lower F/B ratio (F/B= 7,403). Furthermore, the PSR 4 group (severe periodontal disease) showed a higher total bacteria load (mean=23478), and the F/B ratio doesn't show any trend. Concerning, hypertensive individuals they showed no differences in the total bacteria load but showed a higher F/B ratio (F/B=15,37) than not-hypertensive individuals (F/B=10,90). Regarding, diabetic individuals they also don't present differences in the total bacteria load, but again, demonstrated a lower F/B ration (F/B=5.252) than not diabetic (F/B=14,22). All the trends presented show no statistical differences.

Table XI. Quantification means and p-values for 16S all bacteria and *Firmicutes/Bacteroidetes* ratio (number of sequence/DNA ng) for oral and systemic health data ([1]Kruskal-Wallis test; [2]Ordinary one-way ANOVA, [3]t-test).

	16S all bacteria	**16S all bacteria P-value**	***Firmicutes/ Bacteroidetes* ratio**	***Firmicutes/ Bacteroidetes* ratio P-value**
Oral Health				
DMFT[1]				
0-12	22161		7,403	
13-22	12699	0,2642	9,777	0,1724
22-32	14697		17,65	
PSR[2,1]				
0	8940		16,15	
1	17614		2,928	
2	14735	0,4810	11,37	0,7098
3	11124		9,729	
4	23478		10,42	
Systemic condition[3]				
Hypertensive	13979	0,7432	15,37	0,2937
Not-Hypertensive	15195		10,90	
Diabetic	13761	0,6001	5,252	0,2582
Not diabetic	15069		14,22	

Sociodemographic and systemic health data for each individual studied in this section (1.1.1. Saliva microbiome characterization) are shown in Annex 2 and

Annex 3. Microbiome quantification values for each participant are shown in Annex 4. A typical result for *Bacteroidetes* qPCR run is shown in Figure 16. *Firmicutes*, *Bacteroidetes* and all bacteria mean, number of sequences/DNA ng are in Table XII. As expected, when analyzing total bacteria load, *Firmicutes* had a higher percentage (20%) than *Bacteroidetes* (3%) (Table XII). This translates to a relative higher number of sequences/DNA ng of *Firmicutes* when compared to

Bacteroidetes (Figure 17). The mean number of sequences/DNA ng of F/B ratio for the group of study was 12,844.

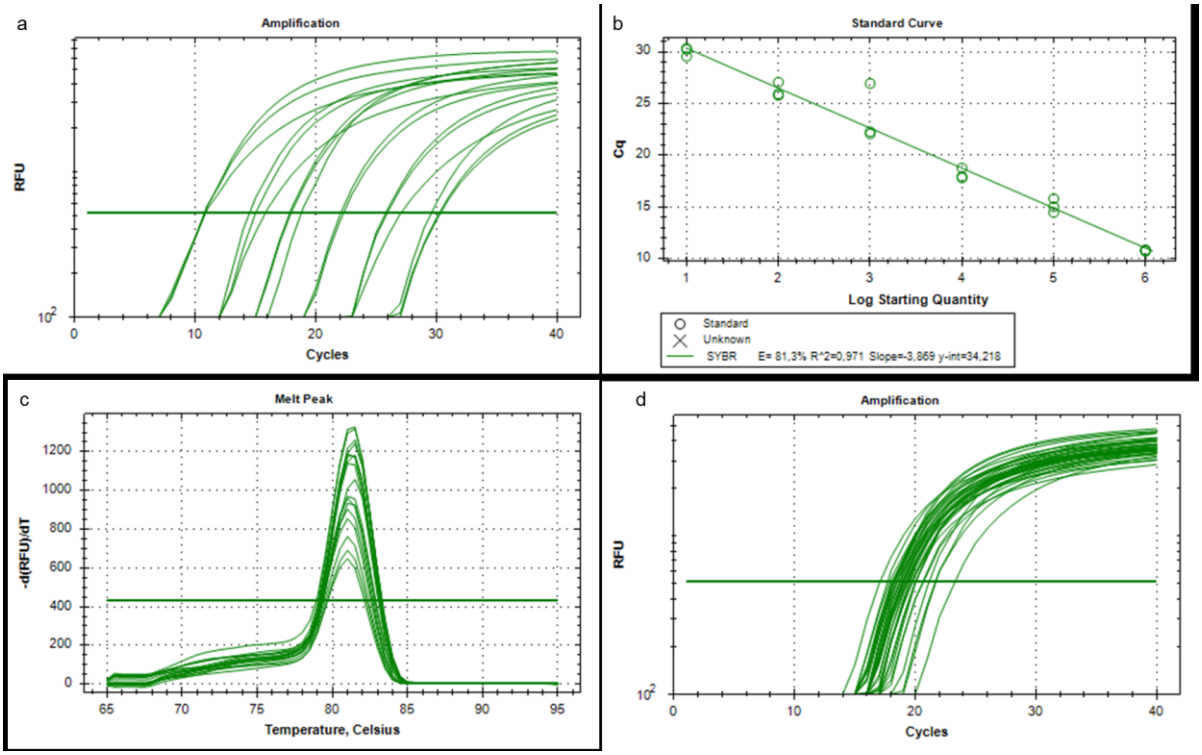


Figure 16. qPCR run results for *Bacteroidetes* phylum: a - standards amplification; b - standard curve; c - standards melting curve, d – samples amplification.

Table XII. Mean number of sequences/DNA ng and percentage of *Firmicutes* and *Bacteroidetes* in all bacteria.

	Mean (Max. – Min.)	**%**
16S all bacteria	10651,058 (33424 - 2200,994)	100
Firmicutes	2179,781 (12409,416 - 10,473)	20
Bacteroidetes	303,032 (4787,88 - 18,774)	3

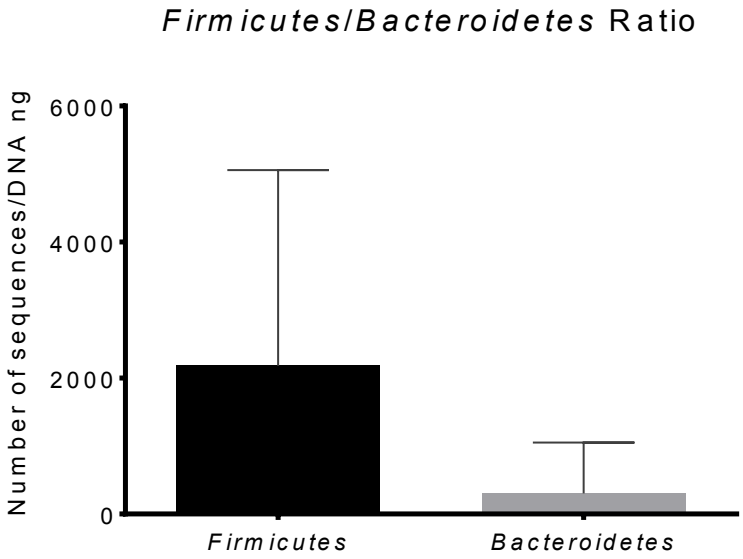


Figure 17. Number of sequences/DNA ng for *Firmicutes* and *Bacteroidetes* phyla.

Discussion

Discussion

Data integration is essential for the holistic understanding of oral health and its relationship with the different factors studied. Oral health clinically assessed, will be related to self-perception, SalivaPrint profiles and microbiome data providing a multidisciplinary approach to oral health. The multiple dimensions evaluated in this work have been established research and experimental strategies in the SalivaTec laboratory and will be continued in the longitudinal study of the Atividade Sénior population of Viseu.

1. Sociodemographic and systemic health

The results of the characterization of this study´s population show that most participants are female even though the percentage (71.5%) is above that observed for the general population (52% in the general population and 58% in the population above 65 years) (102). This is to be expected because females seem to be more receptive to participate in initiatives such as the exercise program from which the population derives. Women in the population under study have a lower mean age than men. From these results we can infer that in terms of age and sex distributions the population studied differs from the Portuguese general population. There are no data specific for the Viseu region and therefore we can´t conclude if our convenience sample is representative of the population over 65 years old of the Concelho.

Most of the participants refer as to not having a Mediterranean diet (65%) which contrary to the results from Schröder, H *et al*. (2011) (103). These authors found that a high adherence to the Mediterranean diet type was associated to a lower BMI and cardiometabolic risk markers (103) which doesn't happen in our study. Participants are mainly from a rural environment and non-smokers or ex-smokers. The very low percentage of smokers (1.48%) is expectable due to the age range of the population and is also in agreement with the decreasing numbers of smokers in the general population which has been a trend of the last decade in Portugal (104).

Regarding systemic health most individuals (85%) refer as not being diabetic and about half (51.94%) as to being hypertensive but 92.6% as to not having cardiovascular disease. These results seem low when compared with data for the Portuguese population (105) where it is estimated that 23.8% are diabetic, 71.3% hipertensive and a litle under 30% die due to cardiovascular disease (106). Our results might be explained by the fact that the data on systemic disease are self reported and often elderly may be uncertain about a specific diagnostic. In future studies it is important that systemic health data can either be provided by the assistant physician or at least confirmed with the health care provider.

An interesting result obtained when the data on diet was analysed considering systemic disease, was the fact that participants which reported as having diabetes had a higher percentage of Mediterranean diet than nondiabetics. This may be a reflex of the higher precautions diabetics have with nutrition and may be an indicator that the nutritional advice given under the “Atividade Senior” program is being followed and results in healthier eating habits.

2. Oral health

DMFT index showed a high mean ± SD (20,82 ± 7,41) when compared to a national study in the same age range, from Calado R. *et al*. (2015) (DMFT=16,17 ± 0,28) (17), suggesting the population sampled in this study has a worse oral health. Nevertheless, according to the Brazilian Health Minister 2003 (107), dental health of our population was satisfactory since the DMFT≤22,17. A study carried out in institutionalized elderly from Viseu, Portugal (14) presented a higher DMFT index (DMFT=26,31 ± 3.79) than our results in non-institutionalized elderly. This could be explained by the higher age average of the institutionalized patients (82,3 yo) (14) and by the fact that it seems that institutionalized patients often present a poorer oral health (108). However, comparing our results with a Brazilian elderly population (DMFT=31,09) our DMFT values are lower (109) which indicates a better oral health regarding caries for our population. Another Brazilian study in an elderly population showed a DMFT index of 29,24 (18) also higher than ours. Other countries however report lower DMFTs than ours. That is the case of Mexico with a DMFT index of 17,2 (22). Therefore, it is obvious that there is room for

improvement in the oral health status of the elderly population in Viseu especially concerning the number of missing teeth.

The indicator that contributed the most to DMFT index was the number of missing teeth which is also observed by Rebelo *et al.* (2016) (18). The fact that the number of treated teeth is lower than the number of missing teeth, can be explained by the age of this population, since exodontia was a common treatment a few years ago when tooth preservation was not considered a priority. Additionally, the fact that the surgical treatment is less expensive than other conservative treatments (10), may prompt many elderly, which are often resource limited, to opt for tooth extraction rather than restoration.

The prevalence of edentulous individuals (11%) was higher than the values reported by the Ordem dos Médicos Dentistas in 2014 (7% edentulous in Portugal) (110). Nevertheless our values are lower than the ones found by Musacchio *et al*. (2007) (44,0% edentulous individuals) in an elderly Italian population (24). The edentulous prevalence (15,5%) of institutionalized elderly from Viseu, Portugal was higher (14) that what we obtained in this study. This is in accordance with the literature which reports that oral health is a concern in institutionalized patients (108). A higher tooth loss among females agreed with previous studies (14,17,19,24,25,111). These results could be explained by the detrimental effect of pregnancy, lactation, and estrogen withdrawal on bone and teeth

The fact that the oral evaluation didn't include an x-ray analysis may be have resulted in the underestimation of filled teeth and overall in the under estimation of the DMFT index. However, the majority of studies (including the ones used for comparison of DMFT indexes) used a similar methodology.

The periodontal status of the population was poor, since 78% of the study population had periodontal disease. It is a higher number than the overall elderly Portuguese population (unhealthy periodontium=71%; including 15% periodontitis) (17). More specifically, our results showed 22% periodontal healthy (but only 3% if edentulous patients are included), 42% slight periodontitis (periodontal pockets 4-5 mm), followed by the 23% moderate periodontitis (periodontal pockets 6-7 mm), 14% severe (periodontal pockets >7 mm). These data when compared with the WHO data from Brazil and China on elderly populations suggests that the periodontal health of our population is similar to theirs (27).

The fact that the periodontal status worsens with age, represents a higher risk for further tooth loss (25). The poor oral health observed can be related to late-life disability (17,25). Nevertheless, Renvert S. *et al*. (2012) (25) reported that a number of under 20 teeth without periodontal compromise could be a good periodontal status indicator. These values could be a goal for the future of our elderly population. However, this implies that the periodontal evaluation strategy includes the recording of the number of periodontal healthy teeth in each individual, and not only the worst tooth in the sextant (PSR index).

Since oral and systemic health are closely connected (112–114), it crucial to monitor oral health in order to provide an early adequate treatment. Concerning, the systemic association with periodontitis only one diabetic didn't show periodontitis, all others had either slight or severe periodontitis, as expected (114). These results indicate that oral health promotion strategies specific for the diabetic population should be implemented in the Atividade Sénior group. It is known that a good oral health is a positive contribution for glycemic control in diabetics (115).

Although the oral health of this sample population is not the worst when compared to results from other countries, there is room for improvement. Towards a better oral health, a more conservative approach implemented in Dental Medicine can contribute to lower the number of loss tooth. Also, the social intervention of dentistry students and dentists, by signaling the individual needs, can contribute to a better oral health of the population of Viseu.

3. Oral health *vs*. self-perception (GOHAI)

The mean GOHAI score for the study population (mean=32,68; high oral health self-perception) was very similar to values (mean=33,1) found by Carvalho C. *et al*. (2013) (57). Although the clinical evaluation of oral health in this population reveals indicators which are below those of other populations, in general self-perception of oral health was satisfactory. This may be explained by the fact that the observed oral conditions are often asymptomatic and individuals do not seek medical treatment. This behavior may result in tooth loss since often, when dental care is sought it is too late to preserve the tooth structure and a more invasive treatment is needed (116). Moreover, comparing our results with Albuquerque 2012 (59), our GOHAI score mean is higher than theirs (mean=17,7). Albuquerque

A. 2012 (59) study population with periodontal disease which were more aware of their oral health condition, showing a lower oral health self-perception. Contrarily, Mohd *et al*. (2017) (117) showed that, for an elderly population from the United Kingdom, the periodontal health had no impact on the quality of life, which is similar to our results. The fact that we found a statistically significant difference in the GOHAI values for individuals under 65 years old, seems to indicate that younger adults have a more accurate perception of their oral health or are more "demanding" with their oral health associated functions.

Students and dentists' intervention in the community is extremely important in order to increase awareness to the oral health importance in the individual wellbeing. Often the elderly are convinced that not having teeth and not being able to properly chew solid foods comes naturally with age and is unavoidable. Periodic generalized oral health assessments are important in order to give patients insightful information about their oral status and refer them for dental treatment as necessary. These general assessments are especially important for populations with less access to proper and frequent dental care. In the long run, this is a way to increase the overall community health status and is an import aspect of community intervention which is part of the mission of higher education institutions and the Institute of Health Sciences-Viseu is no exception.

4. Oral health and the SalivaPrint

SalivaPrint or the total protein electrophoretic profile was used to stratify individuals according to periodontal disease. This strategy enabled the definition of which molecular weight ranges better separate individuals with and without periodontal disease. The molecular weight ranges contributing to total protein profile discrimination correspond to proteins previously implicated in periodontal disease (74). Some of these proteins are functionally related to processes deregulated in oral and systemic disease. Proteins such as P31947 (14-3-3 protein sigma) are related to the mTor pathway involved in glucose resistance, a condition related to obesity and diabetes (118). These two conditions are frequent in the elderly, although as previously stated, in our population self-reported prevalence of diabetes was low.

SalivaPrint showed to be an additional tool, and an important technique useful for individual stratification.

5. Oral and systemic health and the microbiome

The microbiome and host homeostasis is essential for determination of a healthy state. Our results are in agreement with previous studies, since there is a higher number of *Firmicutes* than *Bacteroidetes* (78) in the saliva. Nevertheless, *Firmicutes* and *Bacteroidetes* are in lower amount comparing to a healthy population from Zaura *et al*. (2009) (77). The mean of the ratio *Firmicutes*/*Bacteroidetes* (mean=12,84) was higher than the ratios found by Walter *et al*. (2014) (119) in the gut microbiome.

One of the reasons why there were no statistically significant differences between the variables in study, could be because our group of study was relatively small (only 40 individuals had their SalivaPrint determined). Additionally, studies have also inconsistently related different Firmicutes/Bacteroidetes ratios to different diseases, contrary to what has been observed in experimental settings.

Microbiome studies in human populations reveal that it is difficult to obtain statistically significant differences in microbiome comparisons. In fact, in this study we did not detect any differences in the total bacterial loads or the ratios F/B. We must consider that our sample size was 40 individuals which is substantially lower than what is suggested to see an effect for most microbiome studies (81). Therefore, these results have to be considered as preliminary and supplemented by analysis of further samples both from the collections of this thesis and future works done in the SalivaTec. This will enable the increase in sample size and may reveal statically significant differences. However, it is important to note that the development of this thesis established in SalivaTec the procedure for total bacterial load determination and F/B ratio from saliva samples, paving the way for future studies.

Conclusion

Conclusion

The multidimensional strategy towards the characterization of the oral health in a senior population was fulfilled for the first time in this population. This strategy establishes the benchmarking for subsequent studies.

The main conclusions regarding the population studied are the following:

i) observed population had similar sociodemographic indicators when compared with the Portuguese population except for female/male ratio, and cardiovascular prevalence;

ii) the GOHAI score was relatively high which indicates that this population perceives their quality of life as good;

iii) DMFT index of this senior population (20,8) is higher that the values reported for the Portuguese population, especially for females (22,4);

iv) the number of missing teeth is also higher in females (16,3);

v) periodontal status is poor compared to the values reported for the Portuguese population, a high periodontitis prevalence (22%);

vi) periodontal status is more severe in older individuals.

In addition, with the SalivaPrint analysis we can propose mTOR as a possible biomarker for patients' stratification, as well as, an indicator of some deregulation mechanism in periodontal disease.

As far as the microbiome results and their relation to oral and systemic health, the work on this thesis established the experimental procedures for total bacterial determination as well as for *Firmicutes/Bacteroidetes* ratio in the SalivaTec laboratory. Additionally, it provides preliminary data to be supplemented by further sample analysis in order to obtain sufficient individual numbers for statistical significance.

This thesis provides a multidimensional strategy towards the evaluation of the oral health of a senior population in Viseu. This strategy can be maintained in further studies in order to increase the insight on this population.

Epilogue

With this work, I obtained personal enrichment through the contact with people with different expectations and with different backgrounds. The opportunity of dealing with so many patients was an important experience and enforced the creation of communication strategies between patient and medical staff allowing the enhancement of personal and social experiences. Furthermore, it developed my conflict management skills and was an opportunity to clarify doubts related to clinical conditions and the research project.

This interaction with the Atividade Sénior participants has led to the signaling of individuals with dental treatment needs. On the second visit, we received a positive feedback from individuals which was an indication that they had sought the proper dental treatment. This is a fundamental aspect of the impact this type of work may have on the community. I believe this thesis presents an example of research done with and for the community and reinforces the role of the university in the promotion of oral health in this region.

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Annexes

Annexes

Annex 1. Participants questionnaire.

Questionário de Dadores

1.1. Data de Nascimento_____/_____/_____

1.2. Género
- ❍ Feminino
- ❍ Masculino

1.3. Dados Biométricos
Altura _____ (cm) Peso _____ (Kg) Perímetro Abdominal _____ (cm)

1.4. Etnia
- ❍ Caucasiana
- ❍ Negra
- ❍ Asiática
- ❍ Cigana
- ❍ Outra _____

1.5. Área de Residência
- ❍ Aldeia
- ❍ Vila
- ❍ Cidade

1.6. Estado Civil
- ❍ Solteiro
- ❍ Casado
- ❍ Vive maritalmente
- ❍ Viúvo
- ❍ Divorciado

1.7. Nível de Escolaridade
- ❍ Básico (até ao 9º ano)
- ❍ Médio (até ao 12º ano)
- ❍ Licenciatura, Mestrado e/ou Doutoramento
- ❍ Outro _____

1.8. Profissão _____

2.1 Fuma ou já fumou?
- ❍ Sim
- ❍ Não
- ❍ Ex-fumador

2.2 Se é ex-fumador, há quantos anos deixou de fumar? _____ anos

2.3 Se sim:
Com que idade começou a fumar? _____ anos
Quantos cigarros fuma por dia? _____ cigarros/dia

3.1 Bebe bebidas alcoólicas?
❍ Sim
❍ Não

Se sim, com que idade começou a beber? _____ anos

Frequência do consumo de álcool:
❍ Ocasionalmente
❍ Semanalmente
❍ Socialmente
❍ Diariamente

3.2 Frequência do consumo de álcool:
_____ Nº de copos de vinho / semana
_____ Nº de cervejas / semana
_____ Nº de digestivos / semana

3.3 Deixou de beber?
❍ Sim
❍ Não
3.4 Se sim, há quantos anos? _____ anos

4.1 Grupo Sanguíneo
❑ A
❑ B
❑ AB
❑ O
❑ Rh+
❑ Rh-
❑ Não Sabe

4.2 Toma regularmente medicamentos?
❍ Sim
❍ Não
4.2.1 Se sim, refira-os:_____
_____
_____
_____

4.3 Tomou alguma medicação que não seja indicada acima nos últimos 30 dias?
❍ Sim
❍ Não
4.3.1 Se sim, refira-a: _____
_____

4.4 Tomou algum antibiótico nos últimos 3 meses?
- ❍ Sim
- ❍ Não

4.4.1 Se sim, refira-o:

4.5 Tomou corticosteroides nos últimos 30 dias?
- ❍ Sim
- ❍ Não

4.6 Tomou bifosfonatos nos últimos 30 dias?
- ❍ Sim
- ❍ Não

4.7 Fez a vacina da gripe na última época Outono/Inverno?
- ❍ Sim
- ❍ Não

4.8 Está grávida?
- ❍ Homem
- ❍ Sim
- ❍ Não

4.8.1 Se sim, de quantos meses? _____ meses

4.9 Encontra-se na menopausa?
- ❍ Sim
- ❍ Não

4.9.1 Se sim, há quanto tempo? _____

4.10 Há quanto tempo teve a última menstruação? _____

4.11 Toma anticoncecionais?
- ❍ Sim
- ❍ Não

4.11.1 Se sim, qual? _____

4.12 Nos últimos 12 meses foi consultado por um médico?
- ❍ Sim
- ❍ Não

4.12.1 Se sim, em que especialidade? _____

4.13 Qual a data das últimas análises que efetuou? _____/_____/_____

4.13.1 Foram encontrados valores anormais?
- ❍ Sim
- ❍ Não

4.13.1.1 Se sim, quais? _____
_____

4.14. Tem hipertensão?
- ❍ Sim
- ❍ Não

4.15 Atualmente sofre de alguma efermidade?
- ❍ Sim
- ❍ Não

4.15.1 Se sim, qual? _____

4.15.2 Problemas Cardíacos
- ❑ Doenças das artérias coronárias
- ❑ Angina
- ❑ Arritmias
- ❑ Insuficiência cardíaca
- ❑ Ataque cardíaco
- ❑ Aneurisma da aorta
- ❑ Doença cardíaca congénita
- ❑ Doença cardíaca reumática
- ❑ Outra
- ❑ Não tenho

4.15.3 Diabetes
- ❍ Tipo 1
- ❍ Tipo 2
- ❍ Não tenho

Análise Clínica	**Valores**
Colesterol	
Glicose	
Resistência à insulina	
Hemoglobina glicosilada	
AGEs	
HDL	
LDL	
Triglicéridos	

4.15.4 Doenças auto-imunes:
- ❑ Doença de Crohn
- ❑ Doença de Graves
- ❑ Doença de Behçet
- ❑ Síndrome de Sjogren
- ❑ Outra
- ❑ Não tenho

- ❑ Doenças de sangue. Quais? _____
- ❑ Doenças infeto-contagiosas. Quais? _____
- ❑ Doenças de fígado. Quais? _____
- ❑ Doenças de estômago. Quais? _____
- ❑ Doenças Renais. Quais? _____
- ❑ Epilepsia
- ❑ Asma
- ❑ Urticária
- ❑ Sinusite
- ❑ Acne
- ❑ Outra _____
- ❑ Não tenho

4.16 É alérgico a algum medicamento ou dispositivo médico?
- ❍ Sim
- ❍ Não

4.16.1 Se sim, qual? _____

4.17 É alérgico a algum alimento?
- ❍ Sim
- ❍ Não

4.17.1 Se sim, qual? _____

4.18 É alérgico a picadas de insetos?
- ❍ Sim
- ❍ Não

4.18.1 Se sim, quais? _____

4.19 Foi sujeito a algum tratamento de radioterapia ou quimioterapia?
- ❍ Sim
- ❍ Não

4.19.1 Se sim, há quanto tempo? _____

4.20 História Familiar - Existem doenças na família como?
- ❑ Doenças Cardíacas
- ❑ Diabetes
- ❑ Cancro
- ❑ Não sabe
- ❑ Outras

Questionário Alimentação

5.1. Utiliza azeite como principal gordura culinária? **Sim (1) Não (2)**

5.2. Usa mais de 4 colheres de sopa de azeite por dia? Considere fritar, temperar saladas, refeições fora de casa, etc.). **Sim (1) Não (2)**

5.3. Come mais de 200g (1 porção) de hortícolas por dia? **Sim (1) Não (2)**

5.4. Come mais de 3 peças de fruta por dia (considere um sumo natural como uma peça de fruta. **Sim (1) Não (2)**

5.5. Come menos que uma porção de carne vermelha por dia? Considerar 100-150g de carne ou produtos como presunto, salsicha, fiambre, etc. **Sim (1) Não (2)**

5.6. Come menos que 12g de manteiga, margarina ou natas por dia? **Sim (1) Não (2)**

5.7. Bebe menos que 1 bebida açucarada ou gaseificada por dia? **Sim (1) Não (2)**

5.8. Bebe mais que 7 copos de vinho por semana? **Sim (1) Não (2)**

5.9. Come leguminosas (favas, lentilhas, feijão ervilha, etc.) mais que 3 porções por semana? **Sim (1) Não (2)**

5.10. Come peixe ou marisco (1 dose são 100-150 gramas de peixe ou seja 4-5 unidades ou 200g de marisco) mais que 3 porções por semana? **Sim (1) Não (2)**

5.11. Come bolos ou outros produtos de pastelaria (biscoitos, bolachas, etc.) menos de 3 vezes por semana? **Sim (1) Não (2)**

5.12. Come oleaginosas mais de 3 vezes (nozes, amêndoas, amendoins) por semana? **Sim (1) Não (2)**

5.13. Come preferencialmente frango, peru ou coelho em vez de vaca, porco ou salsichas? **Sim (1) Não (2)**

5.14. Come hortícolas, massa, arroz ou outros pratos com refogados (molho de tomate, cebola, alho francês ou alho e azeite) mais que duas vezes por semana?

Sim (1) Não (2)

Geriatric Oral Health Assessment Index (GOHAI)

Q1 Nos últimos 3 meses diminuiu a quantidade de alimentos ou mudou o tipo de alimentação por causa dos seus dentes?
Sempre Algumas vezes Nunca

Q2 Nos últimos 3 meses teve problemas para mastigar alimentos?
Sempre Algumas vezes Nunca

Q3 Nos últimos 3 meses teve dor ou desconforto para engolir alimentos?
Sempre Algumas vezes Nunca

Q4 Nos últimos 3 meses mudou o seu modo de falar por causa dos problemas da sua boca?
Sempre Algumas vezes Nunca

Q5 Nos últimos 3 meses sentiu algum desconforto ao comer algum alimento?
Sempre Algumas vezes Nunca

Q6 Nos últimos 3 meses deixou de se encontrar com outras pessoas por causa da sua boca?
Sempre Algumas vezes Nunca

Q7 Nos últimos 3 meses sentiu-se satisfeito ou feliz com a aparência da sua boca?
Sempre **Algumas vezes** **Nunca**

Q8 Nos últimos 3 meses teve que tomar medicamentos para passar a dor ou o desconforto da sua boca?
Sempre **Algumas vezes** **Nunca**

Q9 Nos últimos 3 meses teve algum problema na sua boca que o deixou preocupado?
Sempre **Algumas vezes** **Nunca**

Q10 Nos últimos 3 meses chegou a sentir-se nervoso por causa dos problemas na sua boca?
Sempre **Algumas vezes** **Nunca**

Q11 Nos últimos 3 meses evitou comer junto de outras pessoas por causa de problemas na boca?
Sempre **Algumas vezes** **Nunca**

Q12 Nos últimos 3 meses sentiu os seus dentes ou gengivas ficarem sensíveis a alimentos ou líquidos?
Sempre **Algumas vezes** **Nunca**

----------PARE AQUI----------

Status Dentário e Higiene Oral

6.1 Índice CPO

			55	54	53	52	51	61	62	63	64	65			
18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
			85	84	83	82	81	71	72	73	74	45			

Code	Criteria
0	Healthy
1	Carious lesion
2	Filled cavities with carie
3	Filled cavities without carie
4	Lost tooth by carie
5	Lost tooth (by other reason)
6	Sealant
7	Prosthesis or implant
8	Not erupted
T	Trauma
9	Not registered

C ___ P ___ O ___ = _____

6.2 Índice de Higiene Oral - Total _____ (%)

6.3 Periodontal Screening Record (PSR)

1ºQ	2ºQ	3ºQ
6ºQ	5ºQ	4ºQ

Sangramento à sondagem (BOP): _____%

6.4 Diagnóstico Periodontal: _____

Score	Criteria
0	Periodontal health
1	Bleeding on probe (BOP)
2	Dental calculus detected during probing, iaterogenic margin and/or BOP
3	Periodontal pocket 3,5-5.5 mm
4	Periodontal pocket 6 mm or more
*	Periodontal abnormalities present (furcation involvement, tooth mobility, gingival recession, muco-gingival problems)

Observações:

Annex 2. Systemic condition of the individuals included in the microbiome study (N/A - not applied).

Biobanking code	**Gender**	**Age**	**Diet Score**	**Hypertension**	**Diabetes**	**GOHAI score**
D00752	Female	67	8			N/A
D00755	Female	68	8	x		32
D00762	Female	62	12			N/A
D00763	Female	65	7	x		28
D00764	Female	70	8	x		33
D00767	Female	62	9			34
D00772	Female	65	7			36
D00806	Female	60	7	x		N/A
D00823	Female	62	9			N/A
D00832	Male	59	10	x	Type 2	N/A
D00853	Female	68	11		Type 2	N/A
D00855	Female	61	9		Type 2	N/A
D00858	Female	68	9		Type 2	N/A
D00892	Female	71	8	x		36
D00899	Male	65	12			N/A
D00913	Male	73	10			34
D00919	Female	60	11			36
D00920	Male	55	11			33

D00921	Female	56	8			25
D00922	Female	66	9	x		29
D00923	Male	59	10			N/A
D00933	Female	56	7			N/A
D00940	Male	68	8			N/A
D00961	Male	67	10	x		N/A
D00976	Male	65	10			31
D00978	Female	64	9	x		29
D00985	Female	75	8	x		33
D00989	Female	80	9			36
D00991	Female	66	9	x		36
D01007	Male	77	11	x		36
D01018	Female	74	10			N/A
D01020	Female	63	9		Type 2	29
D01048	Male	66	11			36
D01053	Female	64	11		Type 2	35
D01055	Female	76	11			35
D01062	Female	63	9	x		N/A
D01071	Male	68	8	x		34
D01079	Female	61	7			N/A
D01086	Male	68	10	x		30

Annex 3. Oral health of the individuals included in the microbiome study (N/A - not applied).

Biobanking code	**Decayed teeth**	**Lost teeth**	**Filled teeth**	**DMFT**	**PSR**
D00752	3	6	5	14	0
D00755	3	18	0	21	2
D00762	0	8	8	16	2
D00763	3	20	4	27	1
D00764	0	20	5	25	2
D00767	2	23	0	25	2
D00772	6	15	1	22	3
D00806	4	22	0	26	2
D00823	5	21	2	28	4
D00832	5	9	0	14	2
D00853	1	16	1	18	2
D00855	5	17	7	29	4
D00858	0	32	0	32	N/A
D00892	2	9	8	19	3
D00899	3	1	1	5	2
D00913	3	24	4	31	2
D00919	1	14	5	20	2
D00920	3	13	2	18	4
D00921	4	27	1	32	4

D00922	0	4	6	10		0
D00923	8	20	2	28		2
D00933	2	2	0	4		1
D00940	0	32	0	32	N/A	
D00961	1	15	0	16		3
D00976	3	17	2	22		2
D00978	3	9	5	17		4
D00985	2	11	11	24		2
D00989	0	32	0	32	N/A	
D00991	1	26	0	27		3
D01007	2	11	6	19		2
D01018	2	8	2	12		2
D01020	3	10	1	14		0
D01048	1	6	5	12		2
D01053	2	23	0	25		2
D01055	0	32	0	32	N/A	
D01062	2	8	3	13		1
D01071	2	30	0	32	N/A	
D01079	1	21	0	22		2
D01086	3	0	4	7		2

Annex 4. Quantification of 16S all bacteria, *Firmicutes*, *Bacteroidetes* phyla and *Firmicutes*/*Bacteroidetes* ratio (number of sequences/DNA ng).

Biobanking code	**16S all bacteria**	***Firmicutes***	***Bacteroidetes***	**F/B ratio**
D00752		7613,309	184,527	41,259
D00755	2200,994	482,856	59,417	8,127
D00762	10746,719	811,067	181,288	4,474
D00763	4413,449	73,936	18,774	3,938
D00764	16806,662	1279,193	42,498	30,100
D00767	2867,727	2703,689	51,346	52,656
D00772	9080,770	1635,989	203,809	8,027
D00806	15693,638	3508,909	133,354	26,313
D00823	26728,849	3806,668	477,716	7,968
D00832	5059,881	1278,062	152,890	8,359
D00853	13210,314	15,308	173,907	0,088
D00855		1182,413	216,448	5,463
D00858	8169,873	32,618	71,220	0,458
D00892	13166,865	2435,875	105,551	23,078
D00899	26891,156	6198,828	191,901	32,302
D00913	18979,013	2296,062	258,334	8,888
D00919	14118,036	248,536	163,782	1,517
D00920	18164,329	10,473	228,132	0,046

D00921		386,297	155,268	2,488
D00922		2060,563	436,509	4,721
D00923	17594,896	1339,601	211,493	6,334
D00933	24912,027	51,535	293,294	0,176
D00940		12409,416	417,820	29,700
D00961		887,858	155,075	5,725
D00976		431,460	78,262	5,513
D00978	25542,243	6547,537	181,218	36,131
D00985		217,284	85,424	2,544
D00989		4795,432	161,221	29,745
D00991		1255,668	601,588	2,087
D01007	11447,093	627,941	92,988	6,753
D01018		1188,539	290,767	4,088
D01020	8940,230	422,221	171,113	2,467
D01048	5798,524	122,828	44,301	2,773
D01053	33424,709	1810,256	123,344	14,676
D01055	12107,011	1972,265	215,838	9,138
D01062	23515,376	707,287	151,409	4,671
D01071	4882,424	10427,806	154,328	67,569
D01079	9887,974	18,353	94,209	0,195
D01086	31040,487	1717,518	4787,881	0,359