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STUDY OF ORAL MICROBIOTA IN OLDER PEOPLE OF VALENCIA AND ITS RELATION TO AGE, A COGNITIVE IMPARIMENT AND DIET

TRABAJO FIN DE MÁSTER EN BIOTECNOLOGÍA BIOMÉDICA

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RESUMEN

El incremento de la esperanza de vida en el último siglo constituye uno de los grandes logros de la medicina. Pero ello está generando problemas sanitarios, económicos y sociales hasta ahora desconocidos, entre los que se encuentra el alarmante aumento de problemas cognitivos y demencia en personas mayores. La deficiencia cognitiva se ha relacionado con procesos inflamatorios, y estos se perfilarían como el punto común de muchas de las enfermedades asociadas al envejecimiento, como el Alzheimer. En estudios recientes se ha puesto de manifiesto la influencia de la microbiota intestinal y oral en el estado de salud general, y más concretamente en el estado inflamatorio y el nivel cognitivo en el contexto de inmunosenescencia.

Se determinará la relación entre la composición de la microbiota oral, marcadores de tipo inflamatorio y la deficiencia cognitiva en personas mayores de 65 años con distintos grados de nivel cognitivo.

Para ello, se han analizado muestras de saliva de voluntarios de más de 65 años de edad (n=34) con el fin de determinar los perfiles de microbioma mediante técnicas moleculares como PCR cuantitativa dirigida a bacterias, hongos y levaduras, Electroforesis en Gel Gradiente Desnaturalizante-DGGE y secuenciación masiva del gen 16S bacteriano. Se recogieron datos de test cognitivos, así como se analizaron marcadores inflamatorios como la proteina C-reactiva (CRP), myeloperoxidasa (MPO), apoplipoprotiena (APOE) en muestras de sangre y cortisol en muestras de saliva.

En este trabajo se han estudiado las asociaciones entre el microbioma con el grado de deficiencia cognitiva y el índice de masa corporal con el fin de establecer relaciones entre la salud y el envejecimiento saludable.

Nuestros resultados demuestran que grupos bacterianos que se han relacionado en otros estudios previos con las enfermedades periodontales, como los géneros *Prevotella* y *Selenomonas*, aparecen asociados a un menor índice cognitivo y a mayores niveles de mediadores inflamatorios en plasma. Estos resultados apoyan el papel relevante de la inflamación en el desarrollo de las enfermedades neurodegenerativas asociadas con el envejecimiento, así como el impacto de la microbiota oral en estas enfermedades.

Palabras clave: Microbiota bucal, envejecimiento, estado cognitivo

ABSTRACT

The increase in life expectancy in the last century is one of the great achievements of medicine. But it is generating health, economic and social problems, among which is the alarming increase in cognitive problems and dementia in elderly. Cognitive impairment has been linked to inflammatory processes, and these would be the common point of many of the diseases associated with aging, such as Alzheimer. Recent studies revealed the influence that could have the gut and oral microbiota in the general health, and more specifically in the inflammatory and cognitive status in the context of immune senescence.

The relationship between the composition of the oral microbiota, inflammatory markers and cognitive impairment in people over 65 years with varying degrees of cognitive level will be determined.

It has been analyzed saliva samples from volunteers over 65 years of age (n = 34) in order to determine microbiome profiles using molecular techniques such as quantitative PCR directed to bacteria, fungi and yeast, Denaturating Gradient Gel Electrophoresis - DGGE and high throughput sequencing of 16S bacterial gene. Cognitive test data were collected and inflammatory markers in blood samples analyzed as C-reactive protein (CRP), myeloperoxidase (MPO), apolipoprotein E (APOE) and cortisol levels in saliva samples.

We have identified the associations between oral microbiome composition with the cognitive impairment degree and the body mass index in order to establish relationships between health and healthy aging.

Results showed that specific bacterial groups have been linked in previous studies with periodontal diseases, such as *Prevotella* and *Selenomonas* genus, which have been also associated with lower cognitive index and higher levels of inflammatory mediators in plasma. This would support the hypothesis of the role of inflammation in neurodegenerative diseases associated with aging and the possible key role of microbiota in these diseases.

Words: microbiome, aging, cognitive impairment.

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LIST OF ABREVIATIONS

AD: Alzheimer Disease

APO E: Apolipoprotein E

CCF: Compromised cognitive function

DGGE: Denaturing Gradient Gel Electrophoresis

IgA: Immunoglobulin A

IL-6: Interleukin 6

IL-1: Interleukin 1

LPS: lipopolysaccharide

MALT system: Mucosal associated lymphoid system

MMSE: Mini-Mental examination test

MPO: myeloperoxidase

NCF: Normal cognitive function

NF-kB: Nuclear Factor kB

OTU: Operational taxonomic units

PMN cells: Polymorphonuclear cells

q-PCR: Quantitative Polymerase Chain reaction

SCFA: Short Chain Fatty Acids

spp.: Specie

TNF-α: Tumor necrosis factor α

T-reg: Regulatory T cells

1. INTRODUCCTION

1.1 AGING: INMUNOSENESCENCE AND INFLAMMAGING

In developed societies there is a great concern for the health of elderly people. The rapid increase on life-expectancy represents a major challenge and economic burden for modern societies. Due to improvements in medicine and hygiene conditions, it has increased life expectancy of the population, thus increasing the economic cost of health systems by the appearance of aging-associated disease (1). By 2050, elderly population is expected to increase to 1.5 billion, representing 16% of the world's population (2). For all European Union members, it has been estimated that life expectancy for men increased from 77.6 years in 2013 to 84 in 2060 and 83.1 years to 89.1 for women (2). For this reason, it is mandatory to develop new research on these diseases and early detection, as well as treatments to mitigate its effects.

Aging has been defined as a persistent deterioration in the physiological function leading to a lower capacity of response to different stress which is characterized by the onset of associated disease, i.e. osteoarthritis, neurodegeneration, arteriosclerosis, diabetes (3–5). The immune dysregulation is a common characteristic in all those diseases (6,7). Altered immune response with aging may affect the pathophysiology and functionality of the immune system in elderly. This fact is dramatically linked with an increased susceptibility of infections, autoimmune disease or some cancers (8). This phenomenon has been named "**immunosenescence**" and it would play a key role in aging process. On the other hand, elderly people also show a systemic chronic state of low inflammation, known as **"inflammaging**", which is characterized by elevated levels of proinflammatory mediators as interleukines IL6, IL-1 or tumor necrosis factor (TNF-alpha). The inflammaging may be the cause of the increment on the fragility and risk of inflammatory disease in elderly (6,9,10). It has been suggested that an imbalance between pro- and anti-inflammatory mediators may be consequence of the decrease in the Treg activity or the lack of negative feedback from Tcells (11–13). This situation creates an endless cycle in which the inflammation produce a tissue damage with the subsequent production of more proinflammatory mediators (14). The difficulties of setting the causes of inflammation, as well as tissues, organs and components involved make it difficult to design new therapies targeting this process. Further studies are needed to provide light to the complex network of inflammatory mediators involved in the immune response in elderly (15).

There is an intimate interplay between the host immune system and microbiota (16). The mucosal immune system has developed a series of strategies to promote tolerance and avoid creating an inflammatory environment under this bacterial stress that differentiate it from the

general body's immune system (17). The homeostasis between the MALT system (mucosa-associated lympho-reticular tissue) and microbiota support the develop of host tolerance and the control of bacterial burden by an establishment of a constitutive low-grade non-pathological inflammation (18). However, when any component of this delicate relationship is affected, either by disease or simply aging, this state of sub-pathological inflammation could have implications for human health. Thus, the inflammaging would have several sources, including the microbiota which would play a double role (cause/consequence) in the inflammatory process. Currently, efforts are being made to study the possible role of the microbiota in the immunosenescence.

In this respect, we still do not have enough information related to changes and effects caused by aging and its associated diseases with mucosal immunity and its relationship to the microbiota. Thus, a better understanding of human microbiome in parallel with a deeper knowledge on gut mucosal immunity function should be pursued.

1.2 HUMAN MICROBIOTA AND AGING

The set of communities of microorganisms that inhabit different niches of the human body (gut, oral, skin…) is known as microbiota (19). Human microbiota represents a "superorganism" harbouring more cells than human cells and also, more genes than the human genome. Although the exact composition of the microbiota is not exactly known, advances in microbiological techniques, with the emergence of high-throughput sequencing and other new -omics technologies have allowed knowing in greater detail the composition of these microbial communities.

Human gut harbors the majority of these microbial communities, and, correspondingly, has attracted the most research interest. It is estimated that each individual has about 160 species of a consortium of 1,000 to 1,500 (20). However, researchers are interested in the microbiomes that inhabit parts of the body outside the gut, as oral and skin microbiomes.

Accumulating evidence suggest that microbiome is linked to health status and dysbalances in composition have been associated to high risk to develop specific diseases as inflammatory diseases, autoimmune and metabolic problems (21–23).

There are in increasing evidence about the relationship between inflammation and microbiota, but the key players are still unclear. Dysbiosis in microbiome have been described in bowel disease, ulcerative colitis, colorectal cancer, celiac disease, diabetes type 1, obesity,

arteriosclerosis, rheumatoid arthritis, Alzheimer... all of them with a clear inflammatory component in their pathogenesis (24–29). There are several hypotheses about the role of microbiota in establishing this degree of inflammation which is observed in elderly. On the one hand, inflammation may modify microbiota profiles and these changes in microbiota composition may also contribute to the inflammatory status that would be established by many other perpetuate factors (30). However, in this scenario little is known about the role of other microbiomes as oral microbiome in health and disease as well as its impact on the inflammatory diseases. Then, in this paper, we are going to focus on the oral microbiota which is one of the most diverse microbiome in the body resides in the mouth (oral cavity), after gut microbiome in terms of species-richness only behind the gut microbiota.

1.3 ORAL MICROBIOME AND AGING

Oral microbiome is a complex ecosystem formed by a variety of viruses, fungi, archaea and bacteria. It has been identified almost 900 different bacterial species (31,32). The Specific oral microbiota composition is a complicated task due the mouth is an open system, it is exposed to many exogenous factors: food, water, social life… and also, different oral sites (mucosa, tongue, saliva, teeth, oral plaque, etc..) can be analyzed (33). However, the oral microbiome seems to be specific to each person and reminds stable during most of life (34,35).

Referring to the bacterial composition of the mouth, the predominant phyla are Firmicutes, Bateroidetes, Proteobacteria, Actinobacteria, Spirochaetes, Tenericutes and Fusobacteria, which represent 96% of the total bacteria (32) an also uncultured division GNO2, SR1 and TM7 (33) . The human oral microbiome database (HOMD, www.homd.or) is the largest database of taxa descriptions and sequences of oral (36) In case of fungi, Candida species are commonly carried asymptomatically in the middle of individuals and the prevalence increases with age and also inflammatory diseases. However, studies show that the predominant genera in healthy individuals are *Candida, Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium* and *Cryptococcus*. (37).

Archaeas represent a small percentage of all microorganisms of the oral microbiota, with few species, which are all methanogens. As in fungi, their prevalence increased in subjects with oral diseases such as periodontitis (38,39).

Some authors defend that there are similarities inter-individuals at the genus level in the microbiome, but it is more variable at the species levels between individuals. However, it is thought that exist a core oral microbiome common to most individuals (33,40).

Disruption of the oral microbiome has been proposed to mediate on the course of inflammatory disease especially among immunocompromised patients (41). Specific oral microbes have been associated with periodontal health and those that are more prevalent in periodontal disease have been identified (42) . Accumulating data is suggesting the potential link between oral microbiota and the establishment of a proinflammatory state in specific diseases associated with aging, such as atherosclerosis and neurodegenerative diseases (21).

Saliva is crucial in maintaining oral health as it is capable of regulating the bacterial composition, since he possesses antimicrobial agents, such as immunoglobulins, histatins, peroxidases, lysozymes... (43). It is well known that elderly people suffer changes in their diet and hydration, which has repercussions on the production of saliva. It has been observed as in the elderly there is less production saliva (44). This is worsened by inactivity, consumption of certain drugs and reducing hygiene habits, which ultimately conduce to an overgrowth of oral bacteria in elderly. The salivary microbiota is a potential diagnostic indicator of several diseases.For example, a caries-free oral status in have been associated with higher presence of *Neisseria flavescens* and *Porphyromonas catoniae* which have been suggested as oral health markers (45). Higher presence of *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *Streptococcus mitis* in saliva have been linked to high risk of oral cancer (46). In addition, oral microbiome has been also related to obesity as higher levels of *Selenomonas noxia* are associated to obesity in women (47)

1.3.1 Oral microbiome analysis: from traditional methods to Next generation sequencing

There are several techniques to deal with the study of the microbiota, from traditional culture techniques and observational studies to molecular-based methodologies. Around half of oral bacteria are as yet uncultured and culture-independent methods have been successfully used to comprehensively describe the oral bacterial community although little is known yet.

First techniques were based in culture techniques and Gram-staining or microscope examination (48). Since the culture-independent methods introduction, and especially high-throughput techniques, An improvement on the understanding of oral bacterial diversity and showed that 80% of bacteria detected by molecular techniques were not cultivable (49).

Currently, the techniques based on molecular tools are dominant in studies of microbiota, as sequencing, q-PCR (Quantitative-Polymerase chain reaction) and DGGE (Denaturing gradient Gel Electrophoresis). DGGE is a molecular fingerprinting technique of separating the bands of a double stranded DNA by denaturation point which in turn is dependent on the content of GCs to study sequences. This requires a previous amplification so fragments obtained run on a gel with a denaturing gradient. When the double strand is separated, it is fixed in the gel. Thus a produce a fingerprinting of the different studied populations in a given experiment (50). This technique has been used in the study of the composition of archaea in the human microbiota (51).

However, even these new techniques based in the sequence of microorganism, have little resolution with some of the genera present in saliva, as *Actinomyces*, *Streptococcus*, *Neisseria*, *Veillonella*, *Porphyromonas* and *Selenomonas* due to genetic exchange between these genera (52). These discrepancies between the two types of techniques have highlighted the needed to improve culture systems in combination with high sequencing ("Culturomics").

1.3.2 Factors influencing oral microbiome composition

Physicoquimicals factors: temperature and pH

These factors result from the combination of the characteristics of the host, the microbiome and the environment. During the meal, the bacteria are exposed to sudden changes in temperature. However, very few studies have been done on the possible effects of this phenome in the microbiota (53).

The pH of the oral cavity affects the viability and behavior of the microbiota directly as most bacteria do not tolerate extremes pH. However, the pH of the oral cavity usually near neutrality by the effect of saliva. It has been observed as a decrease in pH may promote aciduric bacterial outgrowth, as *Streptococcus mutans* or *Prevotella intermedia* and favor the formation of caries (54.)

Endogenous Factors

Host defense

The first control mechanism of the microbiota is saliva as it is capable of removing large amounts of bacteria from the oral surfaces. It also contains specific defenses such as sIgA and other non-specific factors such as mucins, other glycoproteins, lactoferrin, lysozymes, peroxidases, cystatins and hystatins (55).

Gingival crevice area is controlled by antimicrobial factors of plasma, so there is a continuous flow of gingival fluid with both humoral and cellular components of immune system. It has been observed that in this fluid 90% of the cells are polimorphonuclear leukocytes (PMN) and 10% mononuclear cells (56). These PMN cells are capable of microorganism's phagocytosis and release antimicrobial enzymes such as myeloperoxidase (MPO) which produce reactive oxygen species (ROS). Gingival fluid also contains complement factors that may initiate bacterial cell lysis and IgM, IgG and IgA antibodies.

Age

The oral microbiota is variable during the individual's life, partly due to the change of other factors as eating habits, the state of the teeth, the salivary flow, the immune system ... Since most of these factors are altered in aging, the elderly are highly variable microbiota profiles that rely heavily on their systemic health (53). After birth, oral cavity is exposed to microbes by breathing, breastfeeding, and contact with parents. The pioneers colonizers are Gram-positive cocci, including *Streptococcus* and *Staphylococcus* spp. (57). At three years of age, the salivary microbiome is already complex, but its maturation process continues until adulthood (45). Further studies are needed to identify the oral microbiome development during adult to elderly stage.

Hormonal changes

It has been observed as during puberty and pregnancy are increased gingival inflammation. It has been proposed that this occurs by oral microbiota disturbances produced by hormonal changes. It has been observed the increases in the number of gram-negative anaerobic bacteria as *P. intermedia* in the subgingival microbiota (58,59).

Stress

Stress has been linked to many other factors that affect the microbiota as hormone levels or salivary flow (60,61). One of the major symptoms of stress is increased cortisol levels in plasma and saliva, which in turn can modify the microbiota (62).

Genetic factors

It is known as the genetic background affects the susceptibility of suffering oral diseases such as periodontitis (63). The inheritance of certain immune factors, metabolism, mucus composition and ligand-receptor interactions can affect the selection of certain bacterial groups preferably. However, environmental and social life seem to be more effect than genetic background of the individuals in configuring the microbiota, as prove a study with identical twins (64).

Exogenous factors

Geographical area and diet

Unlike the gut microbiota, which has seen that is strongly influenced by the geographical area of individuals (65) in the oral microbiota it has been observed that these geographical differences are not transmitted in the species present in the saliva (20). It is believed that this is because it is much less influenced by diet and the environment and the individual's own species are more determinant (33).

Exogenous food is not the principal power source of the bacteria in saliva, as it causes the food to be quickly swallowed so food have little time in contact with the microbiota and therefore have no significant effect on its composition (66). Among the exogenous diet, carbohydrates and proteins are the ones that have been linked to changes in the microbiota (67). It has been seen as sucrose rich diets favor the growth of *Streptococcus mutans* and other bacteria associated with caries (68). The fermentation of sugar in lactate lowers the pH, which favors the outgrowth of acidophilic bacteria (66).

Commensal microbiota is known to have important functions in maintaining oral and systemic health. Among these, the colonization of pathogenic species is prevented by simple competition with the commensal microbiota, as fewer spaces available for these species that could cause disease (69) left. This can be seen when the microbiota is altered, for example with the consumption of antibiotics, which favour the appearance of infections such as Candida (70). Besides this, some species have proven to be antagonist to oral pathogens, such as

Streptococcus salivarius which produces a bacteriocin which inhibits the growth of certain pathogenic species associated with halitosis (71).

It seems clear that the variety of bacteria present in the oral microbiota is too large to be studied in it is as a whole and the oral microbiome functionality in terms of metabolic activity would be more important as it was thought. (72)

It has been observed as some bacteria, such as certain streptococci, which have activities glycosidic and endopeptidases (73). Many bacteria have aminopeptidase activity (74) that can ferment amino acids to produce short chain fatty acids. In the gut, these acids have been reported to have benefits on the host, for instance, butyrate is the principal energy source of the colon epithelium and propionate is gluconeogenic (75)). Moreover, some studies show that SCFA have the ability to regulate glucose homeostasis by controlling the release of gastrointestinal hormones (72). The molecular pathways which are responsible for these effects of SCFA, are still unknown.

Oral hygiene and antimicrobial agents

This is one of the most decisive in maintaining the homeostasis of the composition of the microbiota factors. The addition of antimicrobial agents in toothpastes can inhibit bacterial growth of those species associated with oral diseases or reduce expression of virulence factors. Taking oral or systemic antibiotics may also change the composition of the microbiota. It has been observed as after taking antibiotics, the environment can promote oral cavity colonization by other pathogenic species such as *Candida* spp. (53) The placement of intraoral biomaterials, such as dental prostheses or orthodontic appliances, may also induce alterations in the oral microbiome (57). It has been observed that there is an increment of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia*, and *Fusobacterium* species after bracket placement (76).

Oral microbiota and inflammatory disease

Commensal bacteria can metabolize substances or cause to occur by cells which they come into contact and they are discharged into the bloodstream. This could have positive and negative effects on the host's health (15).

Apart from contributing to oral health, it has been suggested that oral microbiota could be related to other systemic diseases, including Alzheimer's disease (AD) (77–79). There is some

evidence linking diseases such as arteriosclerosis, diabetes or obesity with both oral and gut microbiotas.

In their study, Piconi et al. (2009) shown that the reduction of oral anaerobes due to periodontal treatment that changes the habits of hygiene, correlates with a reduction of inflammatory biomarkers and a reversal of thickening of the carotid artery (80).

Animal studies indicate that oral bacteria can cause a premature birth occurs (81). This is supported in humans, where some studies show how mothers suffering from gum disease are more likely to have premature labor or a baby with low birth weight (82). In many of these cases, intestinal and oral bacteria, particularly anaerobes, had crossed the placental barrier (83).

Many pathogens responsible for chronic periodontitis as *Porphiromonas gingivalis*, Treponema forsythia and *Prevotella denticola* are involved in the pathology of inflammatory diseases in distant organs of the oral area. In addition, the first two have been found in the brains of AD patients (84,85). It has also found increased levels of antibodies to periodontal disease-associated as *P. gingivalis* in AD patients compared to controls (86). Other evidence supporting this hypothesis is that the levels of antibodies against oral bacteria, *Fusobacterium nucleatum* and *Prevotella intermedia*, are higher in AD patients compared to controls (87).

In NHANES study, researchers found positive correlations between oral diseases, such as periodontitis and cognitive problems and levels of antibodies against *P. gingivalis* (87,88). *P. gingivalis* has been proposed as a "keystone" in the group of pathogens related to cognitive impairment because its capacity to maintain periodontal associated disease "inflamamophilic" microbiota (89). It has been observed that among the brain consequences of the Lipopolysaccharides (LPS) of *P. gingivalis* are priming of immune cells by differential activation of NF-kB via the Toll Like Receptors (TLRs) (90). This leads to the release of cytokines, complement activation and maintenance of inflammation (91). This bacterium has been linked with cardiovascular disease (92,93), pregnancy outcomes (94–96), rheumatoid arthritis (97,98) and other disease with a common inflammatory component.

The influence of oral microbiota in other disease have been studied, as Diabetes type II and obesity (47,99). For example, it has been detected spirochetes in lesion sites of the pancreatic islet, and it has been speculated that could come from the oral microbiota (100).

Oral microbiota and cognitive deficiencies

Like we mentioned above, world population has a longer life expectancy to our ancestors and as a result, it has increased the prevalence of aging associated diseases, such as neurodegenerative disease. All these diseases have a common feature, an inflammatory component (101,102).

It is believed that this response is maintained by the astroglia releasing proinflammatory cytokines and contributes to maintaining the inflammatory process. That activation of microglia causes neuronal damage which at the same time contribute to maintain inflammation (103,104).

Stimulated microglia produce TNF-a, IL-1, IL-6 and CRP and some studies have correlated the latter and other inflammatory markers with the onset of neurodegenerative disease (105,106). It is thought that glial cells come into contact with blood antigens through routes of entry to the brain, which devoid blood brain barrier. These cells are equipped with TLRs which are able to detect antigens such as LPS for infections elsewhere in the body (79,86,107,108). This hypothesis is supported by studies that have found that there is a greater risk of having an episode of dementia after a systemic infection (107). Numerous studies have shown as TNF levels in the cerebrospinal fluid of neurodegenerative disease patients are much higher than controls (87,102).

Researchers emphasize that the microorganisms, and more specifically the oral microbiota, have a role in the onset or progression of inflammation observed in patients of degenerative diseases (85,91). Miklossy (2011) found that oral bacteria were present in the brains of people with AD in a higher density and variety than in controls (109). Most of these bacteria were oral spirochetes, which are strict anaerobes. In this study, Miklossy and colleagues attempt to demonstrate the ability of spirochetes to reproduce the major hallmarks of AD in vitro and found that there was a significant relationship between spirochetes and AD. In these studies, observed in organotypic cultures as glial and neuronal cells, reproduce the pathological features AD after being exposed to cultures of spirochetes (100). Interactions between such bacteria with perpetual host a chronic inflammation active components of the adaptive and innate immune system, the free radical production, apoptosis, with the production, as mentioned before, the NFTs and amyloid deposition (110).

As we mentioned in the "*Oral microbiota and other inflammatory disease*" section, there is a link between inflammation and some diseases including neurodegenerative problems. All these evidence support the notion that infections may contribute to the maintenance of the inflammatory cycle shown on neurodegenerative pathologies by activating acute phase proteins

and cytokines. The point is that inflammation is maintained in the elderly brains and in this regard, immunogenic proteins and peripheral infections would be a key point in perpetuating this state (91).

1.4 MICROBIOTA-INFLAMMATION-NEURODEGENERATIVE DISEASE LINK

Actually the most accepted theory suggest that a combination of different players as microbiome and immune system have a pivotal role on unbalanced host response favoring a microbial dysbiosis and an aberrant immune system activity(111). Dysbiosis is characterized by an imbalance in the relative abundance of species associated with disease.

An example, *P. gingivalis* is not able to trigger periodontitis disease in germ free mice as a bacterial community is needed for the onset of disease (112). Furthermore, modulation of the host immune system is essential for *P. gingivalis* to have the ability to change the microbiota profile (113,114). It can be concluded that there are many factors, related to the host and microbiota, involved in establishing a profile microbiota altered and proinflammatory state. All these aspects must be taken into account when addressing the research in this field.

Good oral health requires a controlled inflammatory state that can maintain stable microbiota and homeostasis system. However, in diseases, such as periodontitis, this relationship is lost. Whether by a subversion the immune system or host immunoregulatory defects, as often happens in old age (115). This situation can create a self-perpetuating circle of dysbiosis and inflammation (Figure 1).

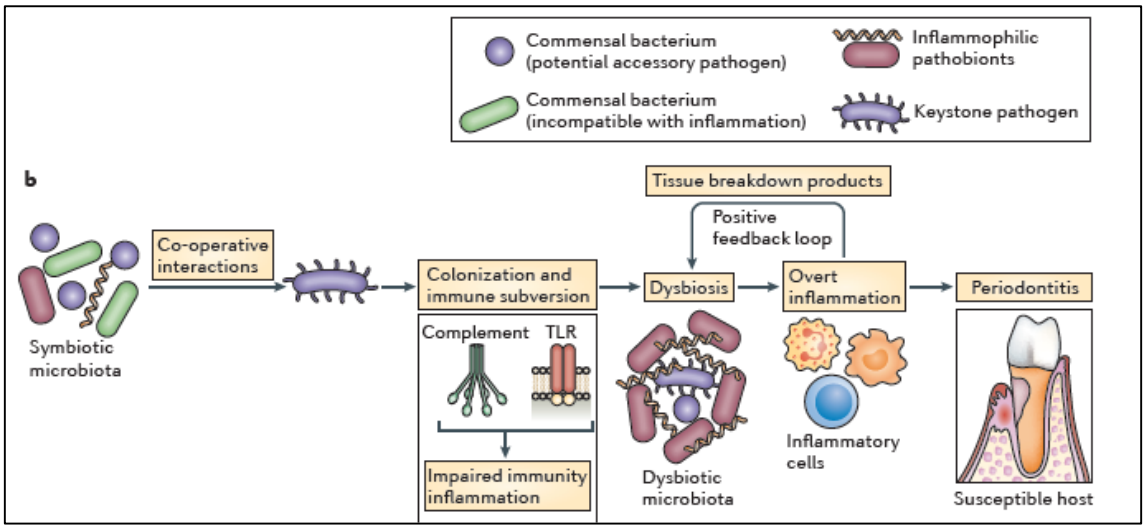

Figure 1. Polymicrobial dysbiosis and inflammation. A self-perpetuating cycle

Many research supports the hypothesis that this inflammation could have effects in systemic health. This is supported by studies that have linked oral bacterial diseases with an increased risk to suffer from diabetes, arteriosclerosis or neurodegenerative diseases (116–118).

To what extent oral microbiome and neurocognitive diseases in elderly processes as inflammaging and immunosenescence remains to be investigated. In this scenario, the aim of this study is to determine whether variations are found in the oral microbiota during elderly and if these are related to cognitive level, immune and nutritional status in old age.

For this purpose, a population –based sample of elderly persons aged 65-80 years oral microbiome was examined and the influence of the cognitive and nutritional status and also, the inflammatory parameters on oral bacteria was discussed.

2. MATERIAL AND METHODS

2.1 Study design and samples

A total of 37 voluntary subjects aged 65 to 80 years from Valencia (Spain) participated in this study.

Biological samples as urine, blood, saliva and fecal samples were collected were kept frozen -20ºC until delivery to the laboratory. Cognitive status was analysed by the use of a cognitive test, Mini mental test examination (MMSE), that classify the volunteers in compromised cognitive function (CCF) and the normal cognitive index (NCF). Other information as medical history, height, weight and other medical data were recorded. The recruitment of volunteers, samples collection and test, both nutritional and cognitive status, were performed by the group led by Dr. J. Enrique de la Rubia, School of Nursing at the Catholic University " San Vicente Ferrer " of Valencia.

2.2 Analysis of inflammatory markers, APOE and cortisol

Enzyme-linked immunosorbent assay (ELISA) was used to quantify levels of C-reactive protein (CRP), myeloperoxidase (MPO) and apolipoprotein E (APOE) in serum according to the manufacturer's instructions (Abcam, Cambridge, UK). Cortisol was measured in saliva with an ELISA assay (Salimetric LLC, Suffolk, UK). MPO is abundantly expressed in neutrophils and is thus used as a marker for inflammation signal.

2.3 DNA extraction

DNA was extracted from buccal swabs, using the Master-Pure DNA extraction Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions with the following modifications: samples were treated with lysozyme (20 mg/mL) and mutanloysin (5U/mL) for 60 min at 37ºC and a preliminary step of cell disruption with 3-µm diameter glass beads followed by 1 min at 2000 oscillations by bead beater (119) . Purification of the DNA was performed using DNA Purification Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions.

2.4 Quantitative analyisis of microbiome by q-PCR

Quantitive PCR amplification and detection were performed with optical-grade 96-well plates with a LightCycler 480 system (Roche, Indianapolis, USA). Region of 16 rDNA from several major groups of microorganisms was amplified using specific primers gathered from the literature (Table 1). All primers were purchased from Isogen Life Science (Utrecht, PW). Each reaction mixture of 10 µl was composed of SYBR green PCR master mix (Roche), 1 µl of each of specific primers at a final concentration of 0.25 µM, and a 1ul of template DNA. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR products from nonspecific PCR products. Standard curves were created using serial 10-fold dilutions of specific fragment amplicon DNA corresponding to 10^2-10^9 gene fragment copies/mg. The concentration of microorganism in each sample was calculated comparing the Ct values obtained from standard curve. The bacterial concentration of each sample was calculated by comparing the threshold cycle (Ct) values obtained from standard curve.

Table 1. Primers used in q-PCR study

Organism	**Primer**	**Sequence (5'-3')**	**Annealing**	**Ref.**
Archaea-Methanobrevibacter Genus	MET-105f MET-386r	TGGGAAACTGGGGATAATACTG AATGAAAAGCCATCCCGTTAAG	55	(120)
Archaea-M. smithii nifH	Mnif-342f Mnif-363r	AACAGAAAACCCAGTGAAGAG ACGTAAAGGCACTGAAAAACC	55	(120)
Fungi	ITS1 F ITS 1 R	TCC GTA GGT GAA CCT GCG G GCTGCGTTCTTCATCGATGC	63	(121)
Total Bacteria	pUniv1 pUniv 2	CGTGCCAGCAGCCGCGGTGGACT ACCAGGGTATCTAATCCTG	60	(122)
***Bacteroides fragilis* group**	g-Bfra-F g-Bfra-R	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	50	(123)
***Bifidobacterium* group**	Bifidum5' Bifidum3'	TGACCGACCTGCCCCATGCTCTG CCCATCCCACGCCGATAGAAT	61	(124)
***Blautia cocoides* group**	g-Ccoc-F g-Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	53	(125)
Enterobacteriaceae	Enterobact-F Enterobact-R	CATTGACGTTACCCGCAGAAGAAG CCTCTACGAGACTCAAGCTTGC	63	(119)

***Enterococcus* group**	Enterococ-R Enterococ F	ACTCGTTGTACTTCCCATTGT CCCTTATTGTTAGTTGCCATCATT	61	(126)
***Lactobacillus* group**	Lacto F Lacto R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	58	(127)
***Staphylococcus* group**	Staphy group F Staphy group R	GGCCGTGTTGAACGTGGTCAAATC A TIACCATTTCAGTACCTTCTGGTAA	58	(128)
***Streptococcus* group**	Strept_1 Strept_2	GTACAGTTGCTTCAGGACGTATC ACGTTCGATTTCATCACGTT	60	(129)
Akkermansia muciniphila	Akker-F Akker-R	CAGCACGTGAAGGTGGGGAC CCTTGC GGT TGG CTT CAG AT	60	(130)
Faecalibacterium spp.	Fprau 07 Fprau 02	CCATGAATTGCCTTCAAAACTGTT GAGCCTCAGCGTCAGTTGGT	60	(131)

For statistical analysis of qPCR results, SPSS software was used. The results were expressed as medians with interquartile ranges (IQR) and non-parametric test were performed due to the non-normal distribution of microbial data. Mann-Whitney U test was used for comparisons between two categories. A p-value <0.05 was considered statistically significant.

2.5 Qualitative analysis of microbiome by Denaturing Gradient Gel Electroforesis (DGGE)

In order to analyze the diversity of bacterial communities present in samples, archaea and fungi, analysis was performed by denaturing gradient gel electrophoresis (DGGE). PCR amplification of the 16S rDNA gene was performed using primers detailed in Table 2. A GC clamp was attached to the 5' of primers F to facilitate the resolution of PCR amplicons by DGGE.

Table 2. Primers used in the DGGE

Target Organism	Primers	Reference
Archaea	0357 F-GC: CCCTACGGGGCGCAGCAG 0691 R: TTACAGGATTTCACT	(132) (133)
Bacteria	357 F: TAC GGG AGG CAG CAG 518 R: ATT ACCGCG GCT GCT GG	(134)
Fungi	NS1: GTAGTCAATATGCTTGTCTC GC fungi: CATTCCCCGTTACCCGTTG	(135)

The PCR protocol included an initial denaturation, a step of annealing and the final extension (the conditions were detailed in Table 3). Each reaction was performed with 5ul of primer (10 uM), 5 ul de Buffer KAPA HIFI Fidelity 5X (0,2 mN de Mg^{+2} 1X), 2 µl de DMSO, 0,5 µl de ADN KAPA HIFI polymerase (1U/µl), 1 µl de dNTPs MIX (10 mM) and 1 µl of DNA. PCR product was visualized by electrophoresis on 1.5% agarose gel stained with red safe to assure that no specific amplification occurs.

Table 3. Conditions for the DGGE analysis

	Archaea (0357F-GC0691 R)		***Bacteria (357F- 518 R)***		***Fungi (ITS-1-GCfungi)***		**Cycles**
Denaturation	6 min	95 °C	3 min	95 °C	4 min	95°C	1
Denaturation	1 min	95 °C	20 seg	95 °C	1 min	95°C	35
Primer hybridization	1min	55 °C	45 seg	55 °C	1 min	50°C	
Extension	2 min	72 °C	1 min	72 °C	1,10 min	2°C	
Final extension	7 min	72 °C	7 min	72 °C	7 min	72°C	1

DGGE was performed on a Dcode Universal Mutation system (Bio-Rad, Hercules, CA). In order to obtain the best discrimination between species, different denaturizing gradients were assay

for each PCR products obtained with the three sets of primers used in polyacrylamide gels [8% (v/v) of a 37.5:1 acrylamide-bisacrylamide mixture]. A 50-70% denaturizing gradient (100% corresponded to 7M urea and 40%) was used for total bacterial gel, a 45-60% denaturizing was used for archaea gel and 10-50% for the fungus gel (136). All of these gradients were made with a gradient maker (Bio-Rad).

A top gel without denaturant was cast above the denaturing gel before the polymerization started. Electrophoresis was run in 1X TAE buffer at 60ºC, for overnight at 100V, after that gels were stained in a SYBR Green solution for 20 min, visualized and photographed under UV.

2.6 Microbiome analysis by 16S RNA Sequencing

Isolated DNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA) and diluted to 5 ng/µL. 16S rDNA gene amplicons were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol. The V3-V4 region of 16s rRNA gene was amplified by PCR using Illumina adapter overhang nucleotide sequences following Illumina protocols. After 16S rDNA gene amplification, the mutiplexing step was performed using using Nextera XT Index Kit. 1 µl of the PCR product was checked on a Bioanalyzer DNA 1000 chip and libraries were sequenced using a 2x300pb paired-end run (MiSeq Reagent kit v3) on a MiSeq Sequencer according to manufacturer's instructions (Illumina). To rule out and control for possible reagent contamination, reagents for DNA extraction and for PCR amplification were also sequenced as controls.

2.7 Bioinformatics and Statistical analysis

Quality assessment was performed by the use of prinseq-lite program (137) applying following parameters: min_length: 50; trim_qual_right: 20; trim_qual_type: mean; trim_qual_window: 20. R1 and R2 from Illumina sequencing where joined using fastq-join from ea-tools suite (138). Data have been obtained using an ad-hoc pipeline written in RStatistics environment (R Core Team, 2012) and data processing were performed using a QIIME pipeline (version 1.9.0) (139).

Chimeric sequences and sequences that could not be aligned were also removed from the data set. The clustered sequences were utilized to construct Operational Taxonomic Units (OTUs) tables with 97% identity and representative sequences were classified into the respective taxonomical level from phylum to genus based on the Greengenes 16S rRNA gene database.

Sequences that could not be classified to domain level, or were classified as Cyanobacteria, were removed from the dataset as they likely represent ingested plant material.

All communities were rarefied to 17,425 reads per sample to calculate bacterial diversity. Subsequently alpha diversity (Shannon and Chao1 index), and beta diversity using UniFrac distance among samples and ADONIS was used to test significance. Calypso version 5.2 (http://cgenome.net/calypso/) was used with data transformed by square root with total sum normalization for the statistical analysis, multivariate test and data mining.

3. RESULTS

3.1 Analysis of inflammatory markers, APOE and cortisol

Cortisol concentration was analysed as a measure of stress levels and it was also obtained the plasma APOE concentration since it has been related with cognitive deficiencies. The inflammatory status was measured by the analysis of the MPO protein and CRP (C- reactive protein) levels.

Inflammation: MPO and CRP concentration

Significant differences (p-value = 0.0015) in the production of MPO between both categories (NCF and CCF) have been found. People with compromised cognitive function (CCF) have increased production of MPO from the innate immune system cells, thus producing a proinflammatory state. No significantly different concentration was found in CRP concentration (Figure 2).

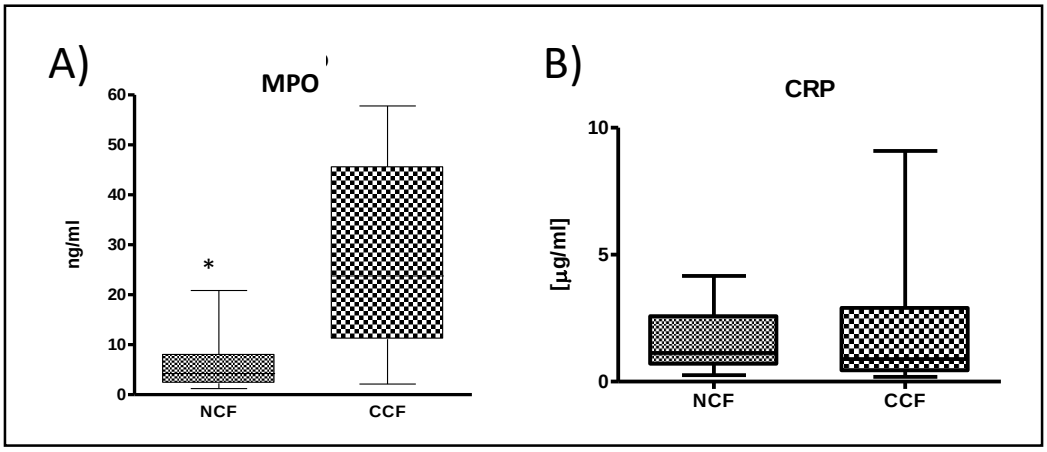

Figure 2. Levels of inflammatory biomarkers of the elderly people with and without cognitive deficiencies. Box plots show the median value (solid line inside boxes), the limits of box represent the 25th and 75th percentile, and whiskers depict the 5th and 95th (p<0.05 CCF vs NCF)*

APOE protein and cortisol levels

No significant difference between the two populations with respect to the concentration of APOE found (p-value = 0.505). However, significant differences (p-value = 0.043) were found regarding the cortisol concentration present in the saliva, being higher in people with cognitive impairments (Figure 3).

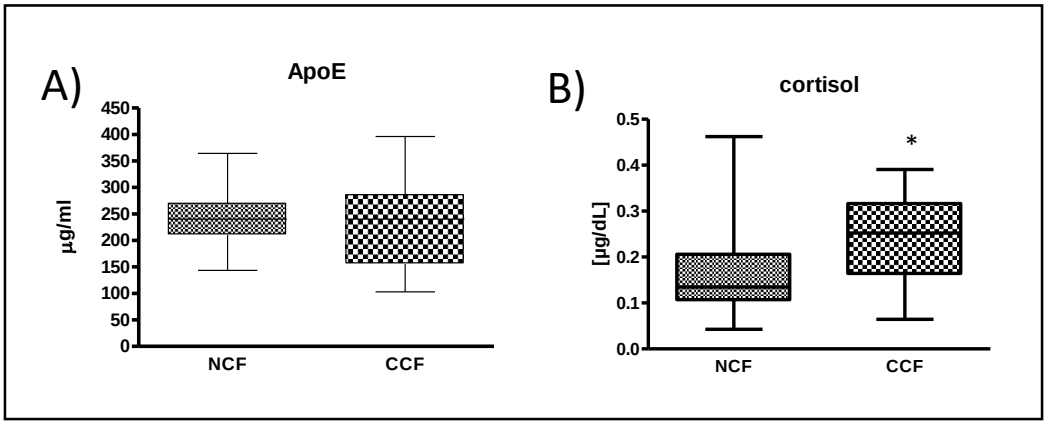

*Figure 3. Concentration of cortisol and ApoE in the both groups of samples. Box plots show the median value (solid line inside boxes), the limits of box represent the 25th and 75th percentile, and whiskers depict the 5th and 95th percentile (*P<0.05 CCF vs. NCF)*

3.2 DGGE profile

Microbial diversity was analyzed by the amplification of the -specific V1-V3- region of the bacterial 16S rRNA, V1-V2 of 18S rRNA of fungi and V3-V4 region of archaea with DGGE primers as described in Table 3. Before performing the gels, amplification was visualized by electrophoresis on an agarose gel (0.8%). We have selected the samples with positive amplification in archaea and fungus for DGGE gels. Bacterial-DGGE profile gel was performed with a sample'randomisation using a sub-set of representative samples from each group. The purpose of this test is to get an overview of the fingerprinting of samples. In future research the identification of possible differential bands between NCF and CCF will be sought. It is possible to see the DGGE gels in supplementary information.

The images of the DGGE gels were digitalized with de Bionumerics software (7.5 version). This program improves image quality by removing the background and determining the position of the bands. It is also able to calculate similarity indexes and cluster samples into a dendrogram based on UPGMA method.

Cluster analysis using the UPGMA dendrogram shown that there were variations in the oral microbiota of samples from the CCF regarding NCF.

Archaea

Archaea DGGE revealed that there are groups banded similar patterns in the ranks of the samples from the elderly with good cognitive index. However, the patterns of the samples of the elderly with cognitive deficits are much more heterogeneous and not grouped as well as the other cluster (Figure 4).

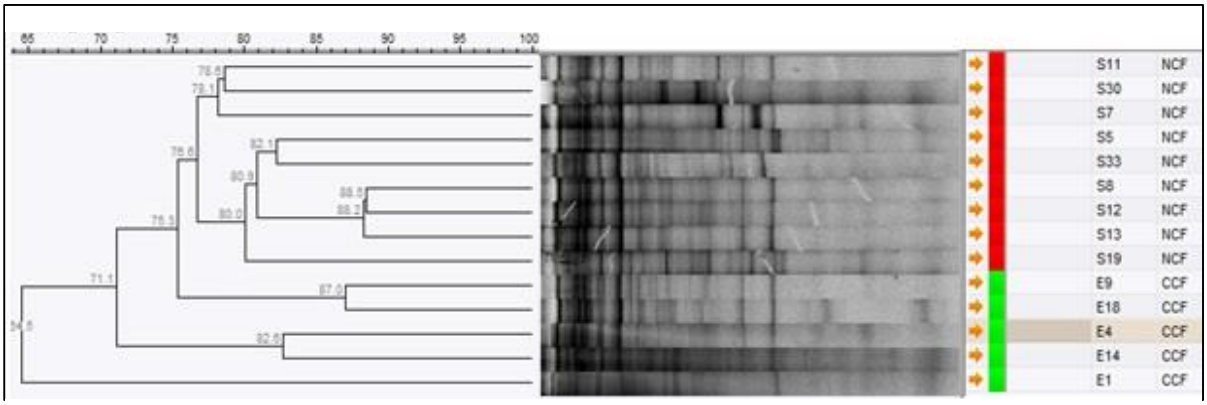

Figure 4. UPGMA trees based on the archaea DGGE results using the Pearson correlation coefficient.

Fungi

In Figure 5 is shown the DGGE gels for fungi PCR products. Although no clear DGGE profile was observed between groups (NCF vs CCF) most of the CCF samples were clustered together showing a subgroup of individuals with similar patterns of fungi.

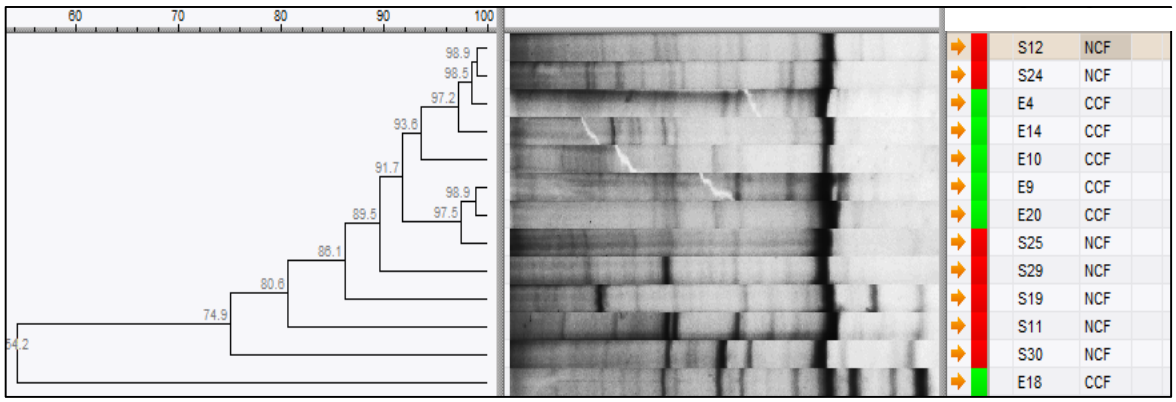

Figure 5. UPGMA trees based on the fungi DGGE results using the Pearson correlation coefficient.

Bacteria

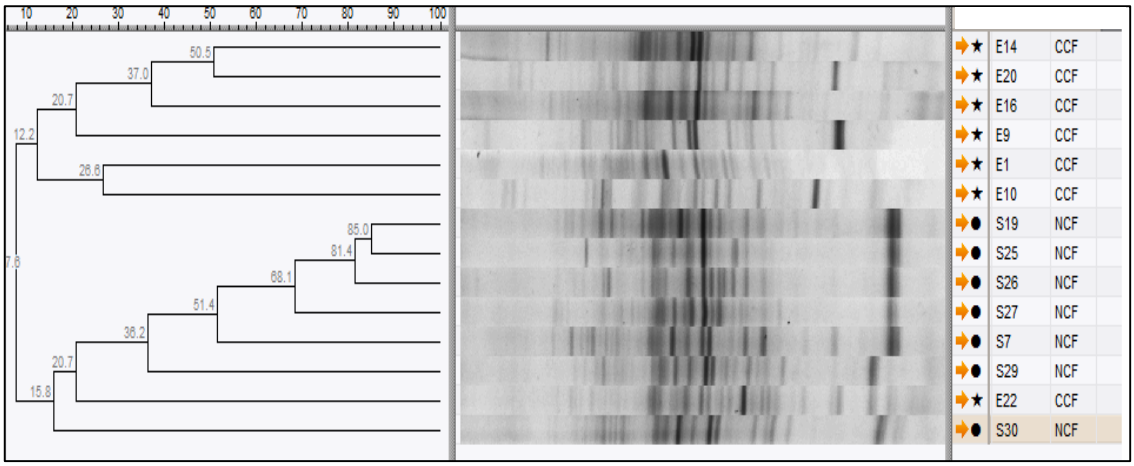

Figure 6. UPGMA trees based on the total bacteria DGGE results using the Pearson correlation coefficient.

In this case, there is a pattern of bands clearly differentiated between samples of the NCF and CCF elderly, so that there were two clusters in the phylogenetic tree.

In general, it has been seen that the NCF -DGGE profiles are much higher homogeneous among them, with more similarity in phylogenetic relationships. While in the case of the CCF, the similarities between the bands are much lower (Figure 6).

It is possible to calculate diversity by using DGGE profiles as rough measure of the diversity of the samples by analyzing the number of bands present in each column of gel (Table 4). It was observed a higher fungal and bacterial diversity and lower archaea diversity in CCF compared to NCF although no significant differences were found between categories in the number of bands in each line of DGGE gel.

Table 4. Number of bands present in each line of DGGE gels grouped by categories, NCF and CCF. Values are expressed as median (interquartile).

Group of organisms	NCF	CCF	Mann Whitney test p-Value
Archaea	29 (27-32)	26 (21.5-39.5)	0.297
Fungi	10 (8-12)	12.50 (8-12)	0.366
Bacteria	18 (16-22)	22 (20-23)	0.053

3.3 Quantitative analysis of oral microbiota by q-PCR

The total content of bacteria in the saliva is around 10^8-10^9 copies/mg of DNA. Higher levels of *Blautia coccoides* (p-value=0.0321) and *Enterococcus* spp. (p-value=0.045) were detected in NCF samples compared to CCF (Table 5).

Table 5. Quantitative analysis of the microbiota profiles in NCF and CCF group by q-PCR

Target	**Log nº copies/mg de DNA**		**Mann-Whitney test**
	NCF (n=14)	CCF (n= 20)	p-value
Methanobrevibacter Genus	4.60 (4.08-4.92)	4.64 (4.489-4.89)	0.500
Total Bacteria	8.90 (8.31-9.03)	8.60 (7.89-8.90)	0.174
Blautia coccoides	8.39 (8.04-8.72)	7.70 (7.36-8.16)	0.032 *
Streptococcus spp	8.10 (7.57-8.38)	7.76 (6.19-8.09)	0.166
Bacteroides spp	4.28 (3.77-4.74)	4.24 (3.10-4.74)	0.785
Enterococcus spp	6.52 (5.89-6.80)	5.94 (5.06-6.21)	0.045 *
***Faecalibacterium* spp.**	3.71 (3.28-4.80)	3.30 (2.58-3.95)	0.200
Enterobacteriaceae family	5.38 (4.84-7.04)	4.87 (4.68-5.14)	0.276

Data are shown as median from positive samples and interquartile ranges (IQR). Statistical analysis was calculated using the Mann Whitney test.

In order to taking account the possible confounding factors, we analyzed the impact of BMI in our sample sets. It was observed higher levels of *Blautia coccoides* group in NCF-normalweight group compared to CCF-normal weight (p-value=0.032) group. No significance differences were found in the other group comparisons.

3.4 Oral microbiota composition by 16S rRNA gene sequencing

The 16S rRNA gene was sequenced from saliva samples of individuals with compromised cognitive function (CCF) and normal cognitive function (NCF). We obtained a mean of 87,412 high quality and classifiable reads of the 16S rRNA gene ranging from 17,425 (minimum) to

143,433 (maximum) per sample (Figure 7). The sequences were clustered with an identity threshold of 97% in “operational taxonomic units” (OTUs). We found a total of 94,781 OTUs. Sequencing data was normalized with a root square transformation, so statistical analysis was made with normal test (ANOVA).

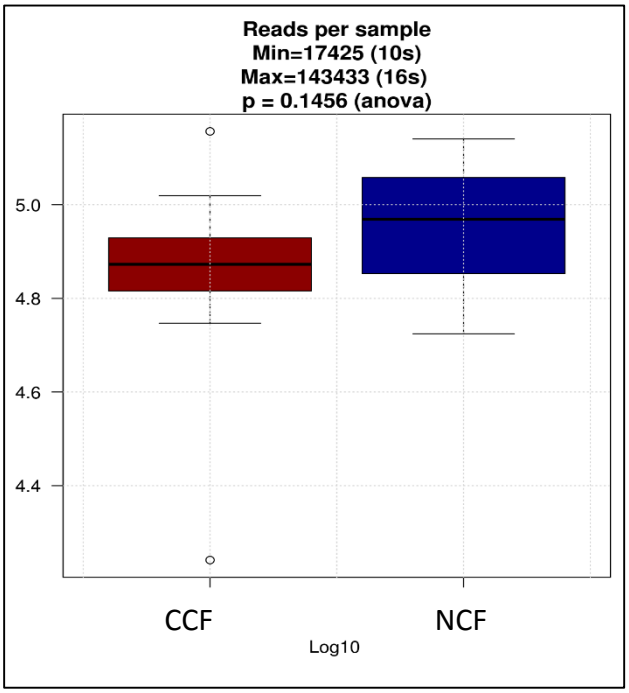

Figure 7. Reads per sample of the CCF (red box) and NCF group (blue box).

Alpha diversity was estimated by different indexes as community evenness (Shannon H) and richness (Chao1) for the two groups of samples (CCF vs NCF). No significant differences were observed. Both Shannon (p-value=0.647) and Chao index (p-value=0.226) differences were not significant, but it is observed that the group of samples CCF tend to have a lower index, and therefore less diversity (Figure 8). To compare the phylogenetic distribution between the cognitive groups, we used Beta-diversity analysis based on UniFrac test (Principal Coordinate Analsyis-PcoA) but not significant differences were found between NCF and CCF (data not shown).

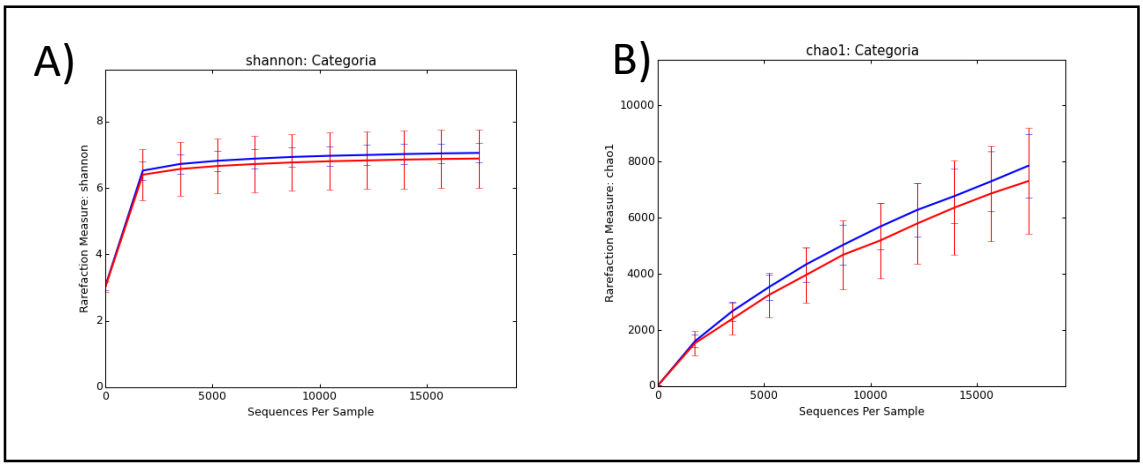

Figure 8. Alpha diversity of the NCF (blue) and CCF (red) group. Shanon index (A) and Chao1 index (B)

Oral microbiota of the samples was dominated by Actinobacteria (15% in NCF and 11% in CCF, p-value=0.405), Bacteroidetes (16% in NCF and 24 % in CCF, p-value=0.232), Firmicutes (44% in NCF and 43% in CCF, p-value=0.906), Proteobacteria (14% in NCF% and 13 in CCF, p value=0.934), Fusobacteria (6% in NCF and 5% in CCF, p-value=0.767) and uncultured group TM7 (3% in both groups, p-value=0.486). In Figure 9 is shown the profiles of relative abundance at phylum levels of each individual. The bar graphs at the family and OTUs level can be consulted in Supplementary information.

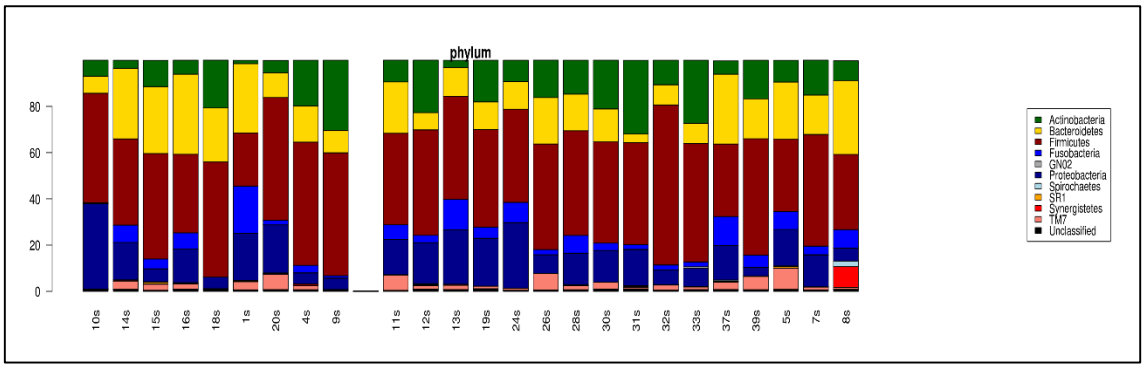

Figure 9. Relative abundance at phylum level of each individual.

It has been found specific families, species and OTUs differed between CCF and CNF groups but there were not differences at phylum level (all p-value<0.05).

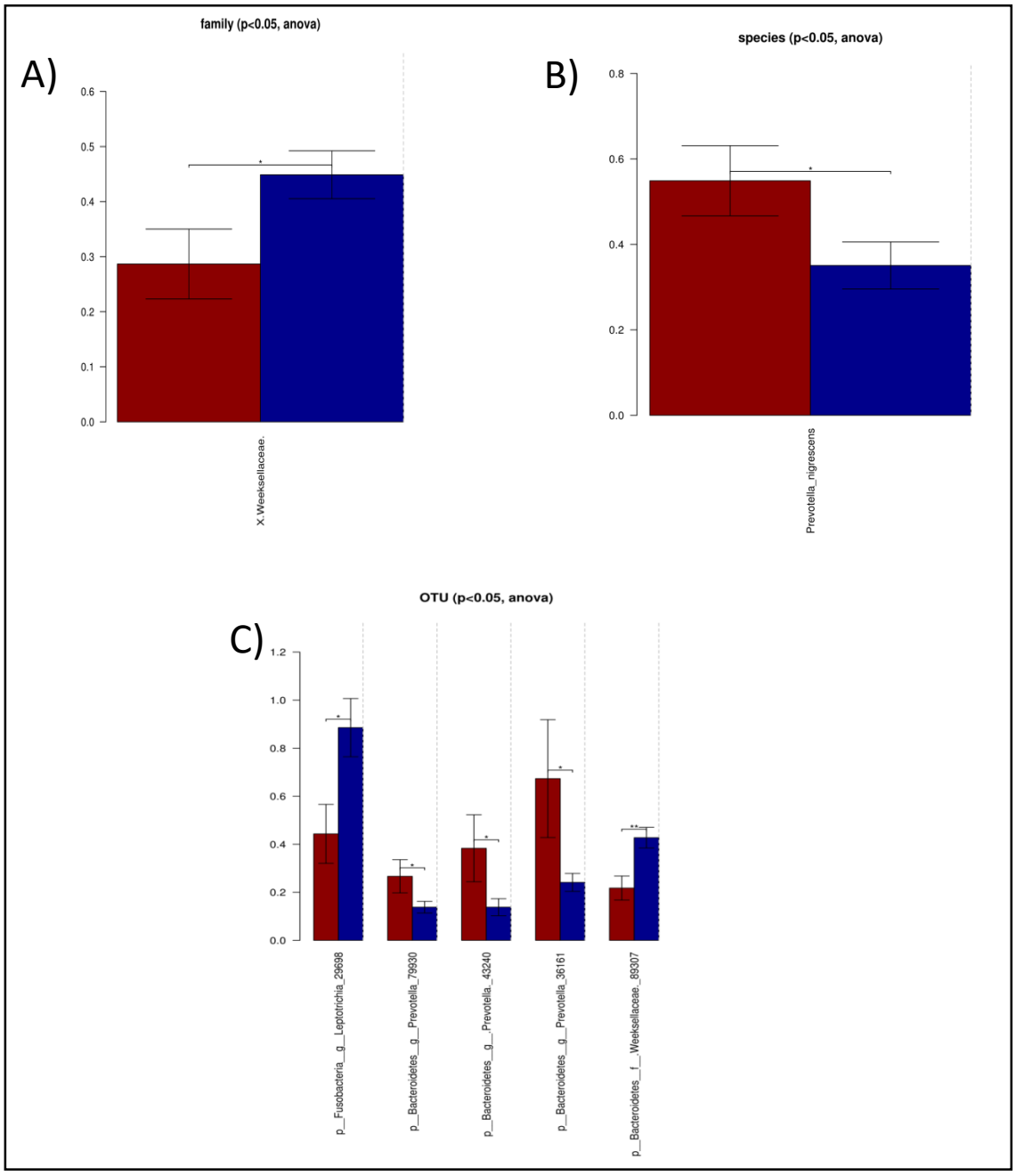

*Figure 10. Family (A), Species (B), and OTUs (C) with significant difference between CCF (red bar) and NCF (blue bar) (*p-value<0.05 CCF vs. NCF).*

Table 6. OTUS with significance difference between NCF and CCF group.

OTUS	p-value	MEAN NCF	MEAN CCF
p__Bacteroidetes__f__.Weeksellaceae._89307	0.0055	0.4275	0.2177
p__Fusobacteria__g__Leptotrichia_29698	0.0270	0.8856	0.4433
p__Bacteroidetes__g__Prevotella_36161	0.0310	0.2412	0.6733
p__Bacteroidetes__g__.Prevotella._43240	0.0410	0.1381	0.3833
p__Bacteroidetes__g__Prevotella_79930	0.0450	0.1381	0.2666
p__Bacteroidetes__g__Prevotella__s__nigrescens_37074	0.0500	0.3431	0.5377
p__Bacteroidetes__g__Prevotella_74219	0.0570	0.0337	0.2166
p__Proteobacteria__g__Haemophilus__s__parainfluenzae_55066	0.0620	0.3756	0.2311
p__Proteobacteria__g__Haemophilus__s__parainfluenzae_9776	0.0730	1.4031	0.8988
p__Bacteroidetes__g__Prevotella_20259	0.0790	0.3781	0.5433

It was observed three OTUs related *Prevotella* spp. to be more represented in people with cognitive impairments compared to NCF. These differences were also significant at specie level, in the case of *Prevotella neigrecens* (p-value = 0.05). However, the OTUS of *Fusobacteria Leptotrichia* was found more represented in NCF (p-value=0.027). At the family level, there was a remarkable difference *X.Weekescella*, which has higher relative abundance in the elderly with normal cognitive function (Table 6).

Relationship between oral microbiome and other parameters

The possible relationship between the presence of certain bacterial groups and an increase in the level of inflammatory markers and cortisol was investigated below. In Figure 11 is shown a heatmap with the variables included in this study and the microbiota at family level. It is possible to found Heatmaps similar for genus and OTUs level in the Supplementary information.

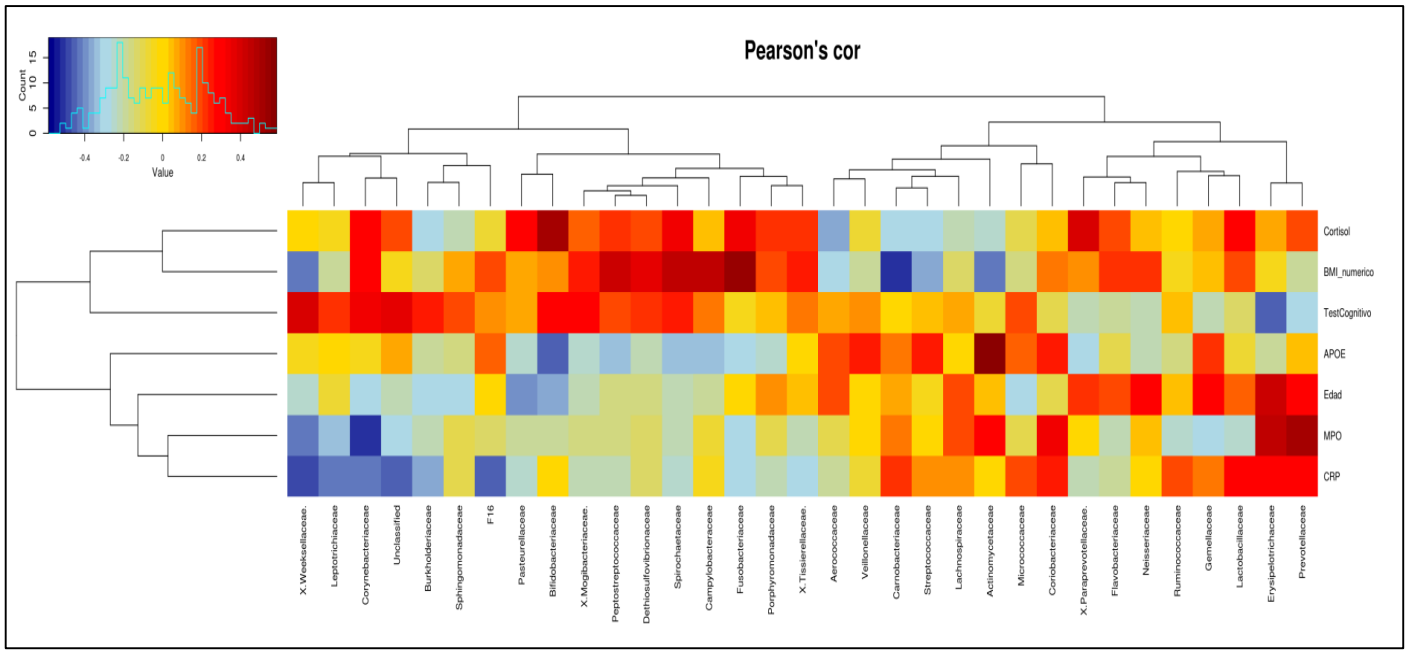

Figure 11. Heatmap of Microbiota and other parameters relationship at family levels.

It has been observed that there are two profiles microbiota. One usually found more related to low inflammation and high cognitive index (left in the Heat map). The other block, much more heterogeneous, were characterized by the presence of families that are more prevalent in older people with high inflammation and low cognitive index (right in the Heatmap). In the opposite direction, it was observed that some species of the *Prevotella* family appeared positively related to high levels of inflammatory mediators and cortisol. Furthermore, this family appeared in individuals with a less cognitive index.

In detail, the correlations observed in the Heat map were analyzed. It was observed that there were specific significant relationships between certain bacterial groups and the parameters studied (Figure 12).

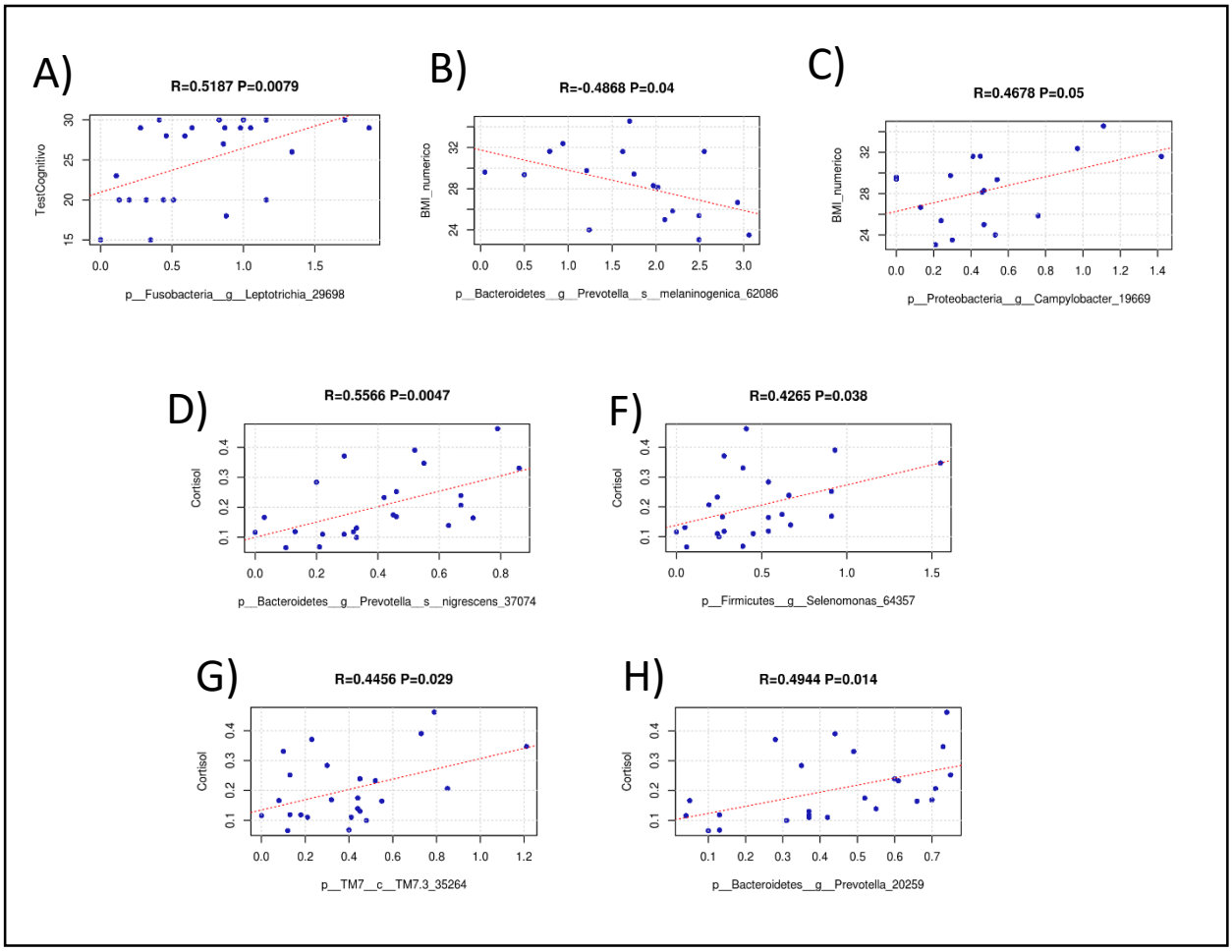

Figure 12. Correlations of the OTUs with parameters studied: Cognitive index (A), body mass index (B-C) and cortisol (D-H).

As shown in the graphs, there was a positive correlation (p = 0.0079) between cognitive index and Leptroticia OTUs, which already showed in significantly different between elderly NCF and CCF.

With respect to BMI, there was a negative correlation with a strain of *P.melangogenica* (p = 0.04) and positive with *Campylobacte*r genus (p = 0.05), so this genus was highly observed in individuals with higher BMI. .

Higher levels of cortisol were related higher presence of four specific OTUs: *Prevotella* (p=0.014), *Prevotella nigrinsens* (p=0.0047), *Selenomonas* (p=0.038) and TM7 (p=0.029). Interestingly, those specific OTUs were highly represented in the group of elderly people with cognitive impairments compare to NCF (Table 6).

Multivariate analysis was performed by conducting a principal component analysis (PCA and bi-plot) (supplementary information). No a clear separation of the two microbiota profiles was found. However, with the graph can be deduced that inflammatory parameters vary in an opposite profile that body mass and cognitive index, which are grouped together. So, the OTUs present in individuals with high inflammatory markers appear with lower relative abundance in high cognitive tests.
With regard to BMI, it is known that this index varies during cognitive deficits, because the elderly with this type of imbalance may lose their appetite, alter their diet or even that this is controlled by health family members (140).

In order to avoid bias or factors that mask the causes of the variation of the microbiota, the study was performed by separating the individuals having a normal weight with respect to more than 25 BMI. A Redundancy analysis (RDA) on normal weight volunteers (BMI <25) was performed (Figure 13). RDA test showed a clearly differentiate groups although it was not significant due the individuals included in the study (n=9).

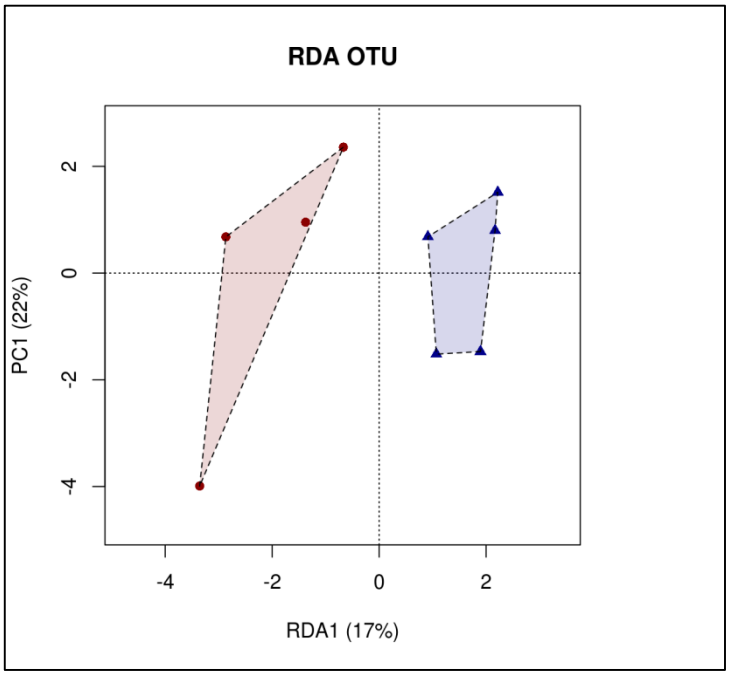

Figure 13. Multivariante analysis (RDA) of the CCF group (red form) and NCF (blue form) group with BMI<25.

With the aim to reduce the impact of BMI variable, we carried out a sub-analysis taking into account normal weight individuals, the study sample is reduced so finding significant differences was more complicated. We observed significant differences in specific OTUs between NCF and CCF groups (Figure 14).

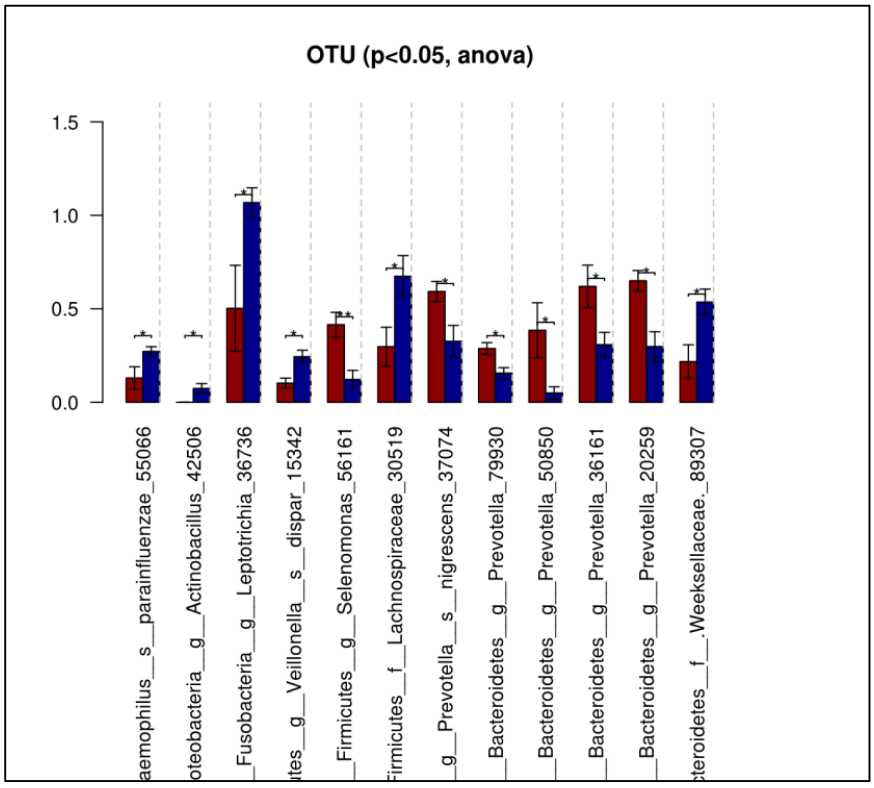

*Figure 14. OTUs with significant difference relative abundant between CCF (red) and NCF group (blue) with BMI<25 (*P<0.05 CCF vs. NCF).*

In normal weight individuals, *Weeksellaceae*, *Leptrotichia* and *Veillonella* OTUS were significantly higher represented in NCF individuals compared to CCF. *Selenomonas* spp*., Prevotella nigrescens* and several OTUs of the genus *Prevotella* (all p-value <0.05) were more frequent in individuals with cognitive impairment (CCF).

When comparing all individuals classified by their cognitive index and its BMI, we obtained similar results. However, OTUs of *Sphingomonas* spp. and *Novosphingobium* spp. appeared closely related with compromise cognitive function individuals (Figure 15).

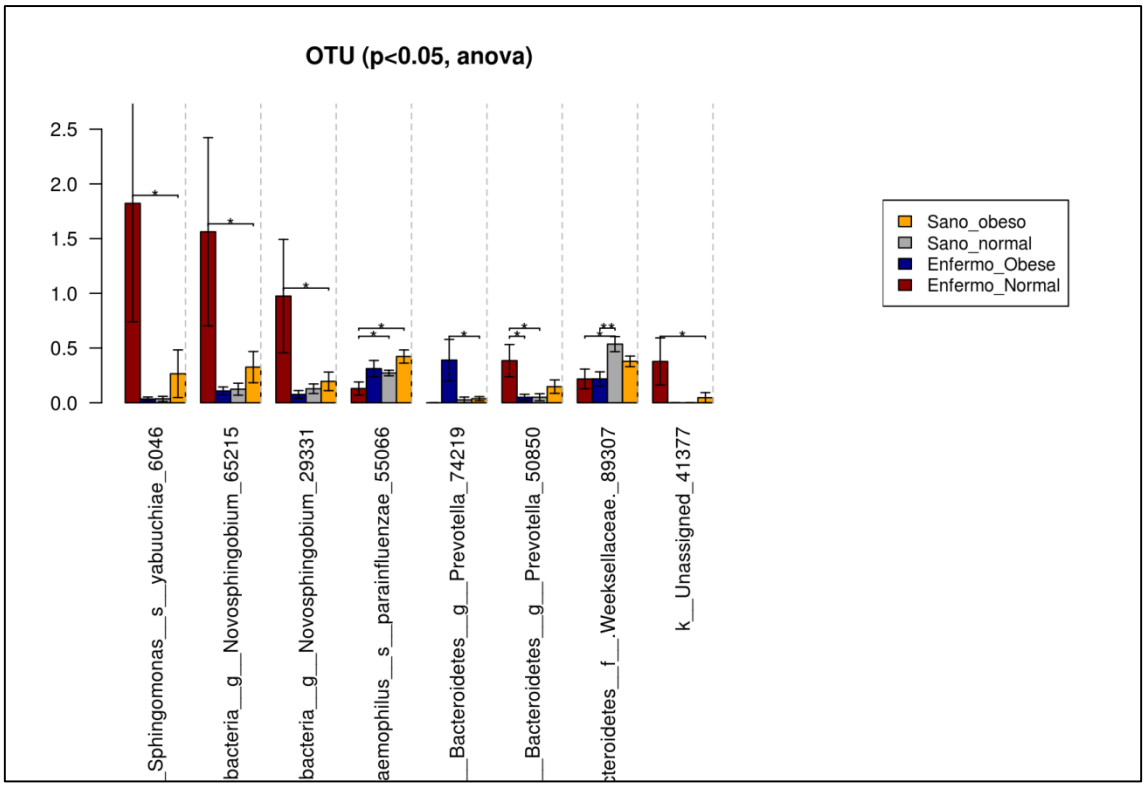

*Figure 15. OTUs significantly different represented in four categories: NCF-normal weight (grey bars), NCF-overweight (yellow), CCF-normal weight (red bar) and CCF-overweight (blue bar) (*P<0.05 CCF vs. NCF).*

If the relationship of the parameters measured in this research with microbiota profile is studied, taking only into account the normal weight individuals, shows that there was a significant positive correlation of cortisol to *Selenomonas* spp. (p-value = 0.005), *Prevotella* spp. (p-value=0.005), *Novosphingobium* spp. (p-value=0.032) and *Sphingomonas* spp. (p-value=0.042). There was a significant negative correlation with *Capnocytophaga* OTUs (p-value=0.0073). Although not significant (p-value = 0.07), there was also a negative correlation *Leptotrichia* spp. with the concentration of cortisol in saliva (Table 7). It is important to note that these were the same OTUs that were significantly more represented in elderly people which have cognitive impairment (see Figure 14).

Table 7. Relationship between cortisol levels and specific microbial features (Pearson correlation)

Taxa	**p-Value**	**R**		**Pr**
p__Firmicutes__g__Selenomonas_64357	0.0050	0.8697	0.628	8
p__Bacteroidetes__g__Prevotella_20259	0.0050	0.8699	0.446	8
p__Bacteroidetes__g__Capnocytophaga_1049	0.0073	-0.8519	0.319	8
p__Proteobacteria__g__Novosphingobium_65215	0.0360	0.7388	0.859	8
p__Proteobacteria__g__Sphingomonas__s__ yabuuchiae_6046	0.0420	0.7239	0.934	5
p__Fusobacteria__g__Leptotrichia_36736	0.0760	-0.6587	0.839	7

The MPO concentration was positively correlated with uncultivable TM7 group (p-value = 0.0028) and *Selenomonas* (p-value = 0.0093) (Table 8). It means that higher levels of MPO were related to higher levels of TM7 and *Selenomonas* in the oral cavity. Although the differences were not significant, it was observed that higher levels of MPO were related to lower abundance of *Veillonella* spp. (p-value = 0.09) and *Leptrotichia spp.* (p-value = 0.120) and higher abundance of specific *Porphyromonas* (p-value = 0.083), *Novosphingosomas* (p-value = 0.180) and *Sphingosomas* (p-value = 0.190). No significant differences were observed in the abundance of any OTUS with CRP levels.

Table 8. Relationship between oral species in microbiota and MPO levels.

Taxa	P.Value	R	MEAN	Pr
p__TM7__c__TM7.3_35264	0.0028	0.8619	0.498	9
p__Firmicutes__g__Selenomonas_64357	0.0093	0.8022	0.607	9
p__Bacteroidetes__g__Prevotella__s__melaninogenica_18153	0.0210	-0.7452	1.054	9
p__Bacteroidetes__g__Porphyromonas_65729	0.0830	0.607	0.346	7
p__Firmicutes__g__Veillonella__s__dispar_5436	0.0990	-0.5833	1.143	9
p__Fusobacteria__g__Leptotrichia_36736	0.1200	-0.5591	0.817	8
p__Bacteroidetes__g__Prevotella_20259	0.1200	0.5549	0.454	9
p__Actinobacteria__g__Actinomyces_46334	0.1800	-0.4912	0.842	9
p__TM7__f__F16_30225	0.1800	0.4861	0.611	8
p__Proteobacteria__g__Novosphingobium_65215	0.1800	0.4877	0.763	8
p__Proteobacteria__g__Sphingomonas__s__yabuuchiae_6046	0.1900	0.4829	0.83	5

DISCUSSION

Progress in high-throughput sequencing has enabled to get a much deeper vision of the complexity of the microbiota present in our body. In addition, these techniques let researchers take a close look at possible changes it undergoes during human diseases and the relationships established between pathological processes and bacterial populations in the different niches of the human body.

In this pilot study, we investigated the possible relationship between changes in the oral microbiota profile and cognitive index in the elderly volunteers. Inflammatory parameters as MPO and CRP levels and also, cortisol were also analyzed as they have been linked to aging-associated diseases which have significant inflammatory component in their pathogenesis.

During typical cognitive decline rate of many neurodegenerative diseases, there has been observed an increase in proinflammatory cytokines such as IL-1, IL-6 and TNF-a, both brain tissue and blood system (105,102).

Myeloperoxidase (MPO) is a myeloid-specific enzyme that generates hypochlorous acid and other reactive oxygen species (ROS) by which it acts as an antimicrobial. This has been found in large concentrations in circulating neutrophils and monocytes, but not usually detectable in the microglia. However, some studies have been reported higher levels of MPO in patients with dementia (101) which suggests that activation of MPO is involved in inflammation pathways observed in neurodegenerative diseases. MPO Activation may cause oxidative stress that has been reported to be involved in Alzheimer development and other inflammatory diseases such as rheumatoid arthritis or atherosclerosis (141,142). In agreement with other studies (143), we have observed a significant increase in the levels of MPO in plasma of people with compromised/impaired cognitive index. Our results are in the agreement with the hypothesis that MPO is having a role in the activation of microglia and the establishment of the proinflammatory state in the pathogenesis of Alzheimer disease.

In our study, we have found that cortisol levels are increased in the elderly volunteers with lower scores on the cognitive tests. Previous studies have linked an increased plasma and cerebrospinal fluid cortisol levels with patients with cognitive deficiencies (144). It has also been found that cortisol levels are associated with compromised memory performance in patients who had mild cognitive impairment (145). Recent data has linked the levels of APOE and cortisol

with a specific molecular signature to diagnose Alzheimer's disease in a prodromal state (146). However, in this study no differences in levels of APOE with respect to NCF were found.

This inflammatory status (high cortisol and high MPO) is related to specific microbial profiles observed in the elderly volunteers who have a compromised cognitive function. Many authors support the hypothesis that microbiota is maintaining the proinflammatory state and in turn this state modulates the composition of the microbial population, creating a feedback circle that encouraging the development of these diseases associated with aging (30).

It was observed a reduction in bacterial diversity without changes in the levels of total bacteria. This fact suggests some bacterial groups are reduced in the elderly patients with compromised cognitive function. These results are in agreement with previous data in other inflammatory disease (147–150). These differential microbial groups would be key for identifying the potential relationships between the oral microbiota and cognitive function. This link has been reported in many studies in elderly patients with inflammatory disease, which could support the hypothesis of the influence of inflammatory state with oral microbiota (111).

It is known the higher inter and intra-individual variability in microbiota and also, how factors as diet, stress, type of life, medication may modulate oral microbiome composition. We also found a high variability in the oral microbiome from elderly volunteers. It is necessary to emphasize the heterogeneity of the elderly population in our study, because there are many variables that differentiate them, such as most have underlying diseases, medication, oral hygiene habits and protests. This study is a pilot study aimed to identify bacterial groups associated to cognitive levels and also, with proinflamatory pathways associated to aging process.

Increased levels of *Blautia cocoides* group (Clostridium *Cluster* XIVa which includes *Clostridium, Blautia, Dorea, Eubacterium, Ruminococcus, Anaerostipes, Roseburia* and *Coprococcus*) by qPCR in the elderly with good cognitive conditions it was observed compared to those ones with compromised cognitive function. *Blautia coccoides* group is a common group in the gut microbiota although some of the member have been also reported in the oral cavity and saliva (151). Recently, it has been reported that *Clostridium* cluster XIV is a normal group in oral cavity in dogs (152) and also, *Faecalibacterium* spp have been described in oral cavity of dogs (152).

Archaea represent a small minority of the oral microbiome, which are restricted to a small number of methanogenic species/phylotypes, namely, *Methanobrevibacter oralis, Methanobacterium curvum/congolense*, and *Methanosarcina mazei* (38). Archaea can be detected in healthy individuals, but its prevalence seems to increase in subjects with

periodontitis and inflammatory diseases. It has been hypothesized that archaea may be involved in syntrophic relationships in the subgingival crevice that promote colonization by secondary fermenters during periodontitis. However, studies with these microorganisms are very scarce. For a long time, *Candida* spp. was the only fungi recognized as part of the normal oral microbial population, despite its opportunistic character (153). However, recent studies had identified 74 genera of cultivable fungi and 11 uncultivable ones in healthy adult oral microbiota (37). Although *Candida* was the most frequent genus, other fungi groups presented a relevant prevalence, such as, *Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium,* and *Cryptococcus*. However, the role of these two organisms, Archaea and fungi, and its identification elderly oral cavity and its role with cognitive related problems is yet to be explored.

For this reason, we analyzed the different microbiota profiles regarding archaea, fungi and total bacteria by denaturing gradient electrophoresis (DGGE). Both fungi and archaea profiles were more heterogeneous in the elderly volunteers with cognitive deficit than samples of controls individuals who have profiles with much greater similarity index. This could be due to the deregulation of the communities that form the normal microbiota, which would lead dysbiosis and establish a profile of the individual. It would be interesting in future studies to identify the organisms responsible for these changes, especially for fungi and archaea, due to its smaller proportion in saliva.

The current trend in the study of the microbiota is to obtain a global vision of all microorganisms that compose it: bacteria, fungi, archaea, viruses. However, very few studies have been conducted on the fungi and archaea population in saliva, possibly due the poor development of genomic databases of these organisms. So the study of differential patterns obtained by DGGE can be a new area of microbiota research.

In agreement with other studies, we found that oral microbiota composed mainly by Firmicutes followed by Bacteroidetes phylum (154). At phylum level, no significant differences were observed between the elderly volunteers classified according to the cognitive index. However, at family and genus levels, we observed some interesting results.

Higher abundance *of Prevotella* family, including *Prevotella nigrescens*, and *Selenomonas* were observed in the CCF group with lower cognitive functions These groups were also related significantly to levels of inflammatory mediators as MPO and high cortisol level, and lower cognitive index .

Bacteroidaeceae family includes *Prevotella* genus which has been traditionally associated with periodontal disease (155,156) and *Porphyromonas* genus, which is considered one of the keystone genres of periodontitis and has been linked to inflammatory diseases, such as arteriosclerosis (21). Species of *Prevotella* genus are capable of producing IgA proteases and colonize the mucosal and teeth surface. For example, *Prevotella intermedia* is able to completely degrade IgA1 and IgA2 (53). *Prevotella nigrescens* has been isolated, together with *P. gingivalis* in necrotic pulps (primary infection) and endodontic lesions (secondary infection) (145). Higher abundance *of Prevotella* family linked to CCF would be related to higher inflammatory status due oral inflammation (periodontitis).

Some species of the *Selenomonas* genus have been also linked to the formation of dental plaque and periodontal disease (157,158). *S. sputigena* is one of the most frequently detected gram negative anaerobic in dental plaque and oral diseases. Other studies have found that oral microbiota of patients with chronic periodontitis is enriched with species of the *Selenomonas* genus (157).

Both genera, *Prevotella* and *Selenomonas*, are considered essentials in the study of periodontal diseases as they are more frequent in this context. In our study these genres are associated with cognitive impairment, which would support the hypothesis that oral microbiota may play a role in the pathogenesis of neurodegenerative diseases, either by participating in the activation of microglia or contribute to an inflammatory state generalized.

It was observed an increased relative abundance of genus *Leptrotichia, Veillonella* and especially the *Weeksellaceae* group in the elderly with full cognitive functions. The family *Weeksellaceae* is found most abundant in elderly with normal cognitive function and lower than those who have a cognitive impairment and inflammatory mediators levels. It is unclear the role of this genus as some studies have been described that *Weeksella virosa* would be the cause of some infectious diseases (159). However, other species of this genus have been isolated from breast milk and infant saliva, being a normal component of the oral microbiota (160). It would be interesting to conduct a study in greater depth in this genre and especially of the functions that can perform these species in the oral cavity.

Leptotrichia genus, part of the normal microbiota of the oral cavity, has been found related to a state of less inflammation and a high cognitive index. The clinical relevance of this group is difficult due culture and identification problems (161). Some researchers have linked *Leptotrichia* genus with oral infections such as gingivitis or periodontitis (42), especially in patients with neutropenia or non-immunocompetent individuals (161). However, other studies

have found that species of this genus were decreased under certain disease conditions. Coit and collegues (2016) conducted a study on Behcet's disease (BD) that causes muco-cutaneous lesions and oro-genital ulcers. They have observed that patients had lower levels of some *Leptotrichia* species (162). The same is observed in other studies as the oral cancer associated microbiota (163) or preterm pregnancies (164).

What happens with *Leptotrichia* genus is usual since most genera have species that are involved in the pathogenesis of some infectious process and, on the other hand, species that are more related to a state of normal health. Moreover, the same species may be included together in a "healthy" microbiota profile and another "unhealthy" profile when it is being studied the same inflammatory diseases. It could be because, in fact, the presence or not of a specific strain or species may be responsible for the dysbiosis, it would be the union of a particular microbiota profile, and not a single species, which wound up in development of the disease or an inflammatory condition. This seriously need some species increase their richness at the same time that others were diminished. This is greatly influenced by individual's own parameters, such as genetics or nutrition. Then, it is necessary to identify possible sources of variation aimed to minimize the variability of the results.

Weight has been described an important factor in elderly (sarcopenia, weight loss, reduced salivary flow...). It has been reported that BMI varied during the development of neurodegenerative diseases, descending this during the first phases of mild cognitive impairment (140). In our study, BMI classification was also studied. Conducting the study with normal weight elderly, new species related to these groups of elders appear as *Veillonella* or *Sphingomonas*. Also *Prevotella* (*P. niegrescens, P. intermedia*) and *Selenomona*s genus have been linked to obesity (47) and yet in our study, continue interacting with the elderly who have less cognitive index, although these have a less BMI. This makes us assume that the relationship established between these two groups is independent of its effect on the obesity. Moreover, when studying only individuals whose BMI is under 25, they continue to be associated with a cognitive impairment.

Removed the BMI variable, it is seen as the *Sphingomonas and Novosphingomonas* genera appear related to the elderly with lower cognitive index. These species are widely distributed in the nature environment and rarely cause serious infections, except in immunosuppressed patients (165,166). *Sphingomonas paucimobilis* is has been related with osteomyelitis, peritonitis and respiratory tract infections (167,168). In addition, some authors have investigated the possible role of *Novosphingomonas* genera with the onset of autoimmune

diseases, such as biliary primary cirrhosis (169). These groups could contribute to the inflammatory state of the individual and this in turn contributes to decreased cognitive functions that have been observed in this study.

Veillonella genus is one of those present in the normal oral microbiota, and its main feature is that use certain metabolites such as lactate which can change the acidity of dental plaque. Some authors suggest that this genus could counteract the formation of dental caries (170,171). Other researchers have found that *Veillonella and Leptotrichia* genera decrease in patients with dysbiosis produced in infections of the upper respiratory tract (154). There have also been researches about the presence of this kind in periodontitis, and has been observed as controls individuals had higher abundance percentages of C*apnocythofaga, Haemophilus, Leptotrichia and Veillonella* (172) than the patients. While individuals with periodontitis had higher levels of *Porphyromonas* and TM7, among others (172).

We found positive correlation with cortisol levels and the same specific OTUs related with the cognitive impairment: *Prevotella, Prevotella niegrescens,* TM7 and *Selenomonas*. This study also shows that an increase of TM7 group is associated with increased levels of MPO. Other authors have found that some members of this division are most often associated with subgingival plaque and oral disease in this area (173). It has also been associated with other inflammatory diseases such as inflammatory bowel disease (174).

It is seen as most groups in this study that are related to increased proinflammatory markers, cortisol and worsening cognitive status of the elderly, have been linked in other studies with periodontal diseases and other inflammatory disease, such as obesity or rheumatoid arthritis. Our results are in agreement with the hypothesis that the change of the composition of the oral microbiota have systemic effects on health of individuals and more specifically in the development of neurodegenerative diseases.

In summary, we it is possible recognize some groups of bacteria that are enriched in elderly who present this profile of inflammation and a compromised cognitive function. The identification of these groups would be very important in addressing the research of the changing the microbiota consequence during illness. These bacterial groups would be interesting for a future research, to discover the effects on the systemic human health.

New microbiological techniques and the massive sequencing have allowed to obtain a vast amounts of information regarding the bacterial composition of the microbiota, opening up a

whole range of possibilities for investigating possible links between microbiota and the development of aging associated disease. However, new challenges arise in this regard, as the combination the information obtained by the different techniques and the need to isolate or avoid the large number of parameters that can be variability sources of variability of the microbiota.

In this study it has been found as genera that have been associated with periodontal disease correlates with lower cognitive scores and with a higher inflammatory state. These same genera have been found in other major diseases such as obesity, arteriosclerosis, arthritis. This would be evidence that the influence of bacterial communities presents in the body in systemic health, being the microbiota an essential factor to considerer in research on such diseases as it would shed light on the pathogenesis, early diagnosis or even treatment of the aging associated disease.

CONCLUSIONS

- Inflammatory markers would be linked to neurodegenerative problems.
- Shifts in bacteria, fungi and archaea would be linked to cognitive status.
- *Prevotella* spp. (*P. niegrescens, P. intermedia*) and *Selenomonas* spp. were associated to lower cognitive functions.
- *Leptrotichia*, *Veillonella* and especially the *Weeksellaceae* group were highly represented in the elderly volunteers with normal cognitive functions.
- Associations with inflammatory markers and specific features of oral microbiome suggest the potential role of bacteria in the neurodegenerative problems in elderly.
- Several factors as diet, life style, oral hygiene and BMI, medicines would act as confounding factors in the elderly oral microbiome study.
- Understanding the links between the microbiome and human disease may provide prophylactic or therapeutic tools to improve human health.

BIBLIOGRAPHY

1. Christensen K, Doblhammer G, Rau R VJ. Ageing populations: the challenges ahead. Lancet. 2009;374(9696):1196–208.

2. Chromorange. The 2015 Ageing Report EUROPEAN ECONOMY 3|2015.

3. Shaw AC, Joshi S, Greenwood H, Panda A LJ. Aging of the innate immune system. Curr Opin Immunol. 2010;22(4):507–13.

4. Rodrigues M. Frailty and cardiovascular risk in community- dwelling elderly : a population-based study. 2014;1677–85.

5. Doyle TA, de Groot M, Harris T, Schwartz F, Stromeyer E, Johnson K, et al. Diabetes, Depressive Symptoms, and Inflammation in Older Adults: Results from the Health, Aging, and Body Composition Study. J Psychosom Res. 2014;75(5).

6. Salvioli S, Monti D, Lanzarini C, Conte M, Pirazzini C, Bacalini MG, et al. Immune system, cell senescence, aging and longevity--inflamm-aging reappraised. Curr Pharm Des. 2013;19(9):1675–9.

7. Haq K, McElhaney JE. Immunosenescence: influenza vaccination and the elderly. Curr Opin Immunol. 2014 Aug;29(1):38–42.

8. Pera A, Campos C, López N, Hassouneh F, Alonso C, Tarazona R, et al. Immunosenescence: Implications for response to infection and vaccination in older people. Maturitas. Elsevier Ireland Ltd; 2015;82(1):50–5.

9. Cevenini E, Monti D, Franceschi C. Inflamm-ageing. Curr Opin Clin Nutr Metab Care. 2013;16(1):14–20.

10. Salazar N, López P, Valdés L, Margolles A, Suárez A, Patterson AM, et al. Microbial targets for the development of functional foods accordingly with nutritional and immune parameters altered in the elderly. J Am Coll Nutr. 2013;32(6):399–406.

11. Tsaknaridis L, Spencer L, Culbertson N, Hicks K, LaTocha D, Chou YK, et al. Functional assay for human CD4 +CD25+ Treg cells reveals an age-dependent loss of suppressive activity. J Neurosci Res. 2003 Oct 15;74(2):296–308.

12. Valiathan R, Ashman M, Asthana D. Effects of Ageing on the Immune System: Infants to Elderly. Scand J Immunol. 2016 Apr;83(4):255–66.

13. Salazar N, Arboleya S, Vald??s L, Stanton C, Ross P, Ruiz L, et al. The human intestinal microbiome at extreme ages of life. Dietary intervention as a way to counteract alterations. Front Genet. 2014;5 (Nov):1–9.

14. Cannizzo ES, Clement CC, Sahu R, Follo C SL. Oxidative stress, inflamm-aging and immunosenescence. J Proteomics. 2011;74(11):2313–23.

15. Callada D, Vianello D, Giampieri E, Sala C, Castellani G, de Graaf A, et al. The role of low-grade inflammation and metabolic flexibility in aging and nutritional modulation thereof: A systems biology approach. Mech Ageing Dev. 2014;136-137:138–47.

16. Kogut MH. The gut microbiota and host innate immunity: Regulators of host metabolism and metabolic diseases in poultry? J Appl Poult Res. Oxford University Press; 2013 Sep;22(3):637–46.

17. Martelli S, Pender SLF, Larbi A. Compartmentalization of immunosenescence: a deeper look at the mucosa. Biogerontology. Springer Netherlands; 2016;17(1):159–76.

18. Candore, G. Caruso, C. Jirillo, E. Magrone, T. Vasto S. Low grade inflammation as a common pathogenic denominator in age-related: novel drug targets for anti-aging strategies and successful ageing achievement. Curr Pharm. 2010;16:584–96.

19. Collado MC, Cernada M, Baüerl C, Vento M, Pérez-Martínez G. Microbial ecology and host-microbiota interactions during early life stages. Gut Microbes. Landes Bioscience; 2012;3(4):352–65.

20. Nasidze I, Li J, Quinque D, Tang K, Stoneking M. Global diversity in the human salivary microbiome. Genome Res. 2009 Apr;19(4):636–43.

21. Hussain M, Stover CM, Dupont A. P. gingivalis in periodontal disease and atherosclerosis - Scenes of action for antimicrobial peptides and complement. Front Immunol. 2015 Feb; (6):1–6.

22. Bahekar AA, Singh S, Saha S, Molnar J AR. The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: a meta-analysis. Am Hear J. 2007;154(5):830–7.

23. Sears CL, Pardoll DM. Perspective: alpha-bugs, their microbial partners, and the link to colon cancer. J Infect Dis. Oxford University Press; 2011 Feb 1;203(3):306–11.

24. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell. Howard Hughes Medical Institute; 2011 May 27;145(5):745–57.

25. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: An integrative view. Cell. Elsevier Inc.; 2012;148(6):1258–70.

26. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A. 2007 Jan 16;104(3):979–84.

27. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med. 2009 Nov 11;1(6):6ra14.

28. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M GW. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14(2):207–15.

29. Maresz KJ, Hellvard A, Sroka A, Adamowicz K, Bielecka E, Koziel J, et al. Porphyromonas gingivalis Facilitates the Development and Progression of Destructive Arthritis through Its Unique Bacterial Peptidylarginine Deiminase (PAD). Kazmierczak BI, editor. PLoS Pathog. Public Library of Science; 2013 Sep 12;9(9):e1003627.

30. Guigoz Y, Doré J, Schiffrin EJ. The inflammatory status of old age can be nurtured from the intestinal environment. Curr Opin Clin Nutr Metab Care. 2008 Jan;11(1):13–20.

31. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol. 2005 Nov;43(11):5721–32.

32. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, et al. The human oral microbiome. J Bacteriol. 2010 Oct;192(19):5002–17.

33. Wade WG. The oral microbiome in health and disease. Pharmacol Res. Elsevier Ltd; 2013;69(1):137–43.

34. Yamanaka W, Takeshita T, Shibata Y, Matsuo K, Eshima N, Yokoyama T, et al. Compositional Stability of a Salivary Bacterial Population against Supragingival Microbiota Shift following Periodontal Therapy. White BA, editor. PLoS One. Public Library of Science; 2012 Aug 16;7(8):e42806.

35. Rasiah IA, Wong L, Anderson SA SC. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch Oral Biol. 2005;50(9):779–87.

36. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, et al. Structure, function and diversity of the healthy human microbiome. Nature. Nature Publishing Group; 2012 Jun 13;486(7402):207–14.

37. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the Oral Fungal Microbiome (Mycobiome) in Healthy Individuals. May RC, editor. PLoS Pathog. Public Library of Science; 2010 Jan 8;6(1):e1000713.

38. Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, Relman DA. Methanogenic Archaea and human periodontal disease. Proc Natl Acad Sci U S A. National Academy of Sciences; 2004 Apr;101(16):6176–81.

39. Matarazzo F, Ribeiro AC, Feres M, Faveri M, Mayer MPA. Diversity and quantitative analysis of Archaea in aggressive periodontitis and periodontally healthy subjects. J Clin Periodontol. 2011 Jul;38(7):621–7.

40. Zaura E, Keijser BJ, Huse SM, Crielaard W, Turnbaugh P, Hamady M, et al. Defining the healthy "core microbiome" of oral microbial communities. BMC Microbiol. BioMed Central; 2009;9(1):259.

41. Beumont MG, Duncan J, Mitchell SD, Esterhai JL, Edelstein PH. Veillonella Myositis in an Immunocompromised Patient. Clin Infect Dis. Oxford University Press; 1995 Sep 1;21(3):678–9.

42. Colombo AP V, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, et al. Comparisons of subgingival

microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. J Periodontol. 2009 Sep;80(9):1421–32.

43. de Almeida PDV, Grégio AMT, Machado MAN, de Lima AAS, Azevedo LR. Saliva composition and functions: a comprehensive review. J Contemp Dent Pract. 2008;9(3):72–80.

44. Navazesh M, Mulligan RA, Kipnis V, Denny PA, Denny PC. Comparison of whole saliva flow rates and mucin concentrations in healthy Caucasian young and aged adults. J Dent Res. 1992 Jun;71(6):1275–8.

45. Crielaard W, Zaura E, Schuller AA, Huse SM, Montijn RC, Keijser BJ. Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. BMC Med Genomics. 2011;4(1):22.

46. Mager DL, Haffajee AD, Devlin PM, Norris CM, Posner MR, Goodson JM. The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. J Transl Med. 2005;3:27.

47. Goodson JM, Groppo D, Halem S, Carpino E. Is obesity an oral bacterial disease? J Dent Res. 2009;88(6):519–23.

48. Lagier JC1, Armougom F, Million M, Hugon P, Pagnier I, Robert C, Bittar F, Fournous G, Gimenez G, Maraninchi M, Trape JF, Koonin EV, La Scola B RD. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin Microb Infect. 2012;18(12):1185–93.

49. Lagier J-C, Million M, Hugon P, Armougom F, Raoult D. Human gut microbiota: repertoire and variations. Front Cell Infect Microbiol. Frontiers Media SA; 2012;2:136.

50. Carmona M, Sepúlveda D, Cárdenas C, Nilo L, Marshall SH. Denaturing gradient gel electrophoresis (DGGE) as a powerful novel alternative for differentiation of epizootic ISA virus variants. PLoS One. Public Library of Science; 2012;7(5):e37353.

51. Hugon P, Lagier J-C, Colson P, Bittar F, Raoult D. Repertoire of human gut microbes. Microb Pathog. 2016; 4010(15)

52. Hanage WP, Fraser C, Spratt BG. Fuzzy species among recombinogenic bacteria. BMC Biol. 2005;3:6.

53. Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev. 1998;62(1):71–109.

54. Baliga S, Muglikar S, Kale R. Salivary pH: A diagnostic biomarker. J Indian Soc Periodontol. Medknow Publications; 2013 Jul;17(4):461–5.

55. Rudney J, Krig M, Neuvar E, Soberay A, Iverson L. Antimicrobial proteins in human unstimulated whole saliva in relation to each other, and to measures of health status, dental plaque accumulation and composition. Arch Oral Biol. 1991;36(7):497–506.

56. Buchmann R, Hasilik A, Nunn ME, Van Dyke TE, Lange DE. PMN responses in chronic periodontal disease: evaluation by gingival crevicular fluid enzymes and elastase-alpha-1-proteinase inhibitor complex. J Clin Periodontol. Munksgaard International Publishers; 2002 Jun;29(6):563–72.

57. Sampaio-Maia B, Monteiro-Silva F. Acquisition and maturation of oral microbiome throughout childhood: An update. Dent Res J (Isfahan). Medknow Publications; 2014 May;11(3):291–301.

58. Markou E, Eleana B, Lazaros T, Antonios K. The influence of sex steroid hormones on gingiva of women. Open Dent J. Bentham Science Publishers; 2009;3:114–9.

59. Fischer CC, Persson RE, Persson GR. Influence of the menstrual cycle on the oral microbial flora in women: a case-control study including men as control subjects. J Periodontol. 2008 Oct;79(10):1966–73.

60. Bergdahl M, Bergdahl J. Low unstimulated salivary flow and subjective oral dryness: association with medication, anxiety, depression, and stress. J Dent Res. 2000 Sep;79(9):1652–8.

61. Naumova EA, Sandulescu T, Bochnig C, Khatib P Al, Lee W-K, Zimmer S, et al. Dynamic changes in saliva after acute mental stress. Sci Rep. Nature Publishing Group; 2014 May 8;4:239–45.

62. Gur TL, Worly BL, Bailey MT. Stress and the commensal microbiota: importance in parturition and infant neurodevelopment. Front psychiatry. Frontiers Media SA; 2015;6:5.

63. Michalowicz BS. Genetic and heritable risk factors in periodontal disease. J Periodontol. 1994 May;65(5 Suppl):479–88.

64. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, et al. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res. 2012 Nov;22(11):2146–52.

65. Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, et al. Differences in Fecal Microbiota in Different European Study Populations in Relation to Age, Gender, and Country: a Cross-Sectional Study. Appl Environ Microbiol. American Society for Microbiology; 2006 Feb 1;72(2):1027–33.

66. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res. 2011 Mar;90(3):294–303.

67. RUNNEL R, SAAG M, OLAK J, HONKALA E, HONKALA S, MKINEN K, et al. Changes in Oral Microbiota after Long-term Daily Sugar Alcohols Using. 2014;

68. Wennerholm K, Birkhed D, Emilson CG. Effects of sugar restriction on Streptococcus mutans and Streptococcus sobrinus in saliva and dental plaque. Caries Res. 1995;29(1):54–61.

69. Vollaard EJ, Clasener HA. Colonization resistance. Antimicrob Agents Chemother. American Society for Microbiology (ASM); 1994 Mar;38(3):409–14.

70. Sullivan, A., Edlunc, C., Nord C. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis. 2001;1(2):101–14.

71. Wescombe PA, Heng NCK, Burton JP, Chilcott CN, Tagg JR. Streptococcal bacteriocins and the case for Streptococcus salivarius as model oral probiotics. Future Microbiol. 2009 Sep;4(7):819–35.

72. O'Connor EM, O'Herlihy E a, O'Toole PW. Gut microbiota in older subjects: variation, health consequences and dietary intervention prospects. Proc Nutr Soc. 2014; 1–11.

73. Hulting G, Flock M, Frykberg L, Lannergård J, Flock J-I, Guss B. Two novel IgG endopeptidases of Streptococcus equi. FEMS Microbiol Lett. 2009 Sep;298(1):44–50.

74. Suido H, Nakamura M, Mashimo PA, Zambon JJ, Genco RJ. Arylaminopeptidase activities of oral bacteria. J Dent Res. 1986 Nov;65(11):1335–40.

75. Salazar N, Arboleya S, Valdés L, Stanton C, Ross P, Ruiz L, et al. The human intestinal microbiome at extreme ages of life. Dietary intervention as a way to counteract alterations. Front Genet. Frontiers Media SA; 2014;5:406.

76. Al-Anezi SA. Dental plaque associated with self-ligating brackets during the initial phase of orthodontic treatment: A 3-month preliminary study. J Orthod Sci. Medknow Publications; 2014 Jan;3(1):7–11.

77. Ahn J, Chen CY, Hayes RB. Oral microbiome and oral and gastrointestinal cancer risk. Cancer Causes Control. 2012 Mar;23(3):399–404.

78. Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, et al. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. Gut. 2012 Apr;61(4):582–8.

79. Holmes C, El-Okl M, Williams AL, Cunningham C, Wilcockson D, Perry VH. Systemic infection, interleukin 1beta, and cognitive decline in Alzheimer's disease. J Neurol Neurosurg Psychiatry. 2003 Jun;74(6):788–9.

80. Piconi S, Trabattoni D, Luraghi C, Perilli E, Borelli M, Pacei M, et al. Treatment of periodontal disease results in improvements in endothelial dysfunction and reduction of the carotid intima-media thickness. FASEB J. 2009 Apr;23(4):1196–204.

81. Fardini Y, Chung P, Dumm R, Joshi N, Han YW. Transmission of diverse oral bacteria to murine placenta: evidence for the oral microbiome as a potential source of intrauterine infection. Infect Immun. American Society for Microbiology (ASM); 2010 Apr;78(4):1789–96.

82. Dörtbudak O, Eberhardt R, Ulm M, Persson GR. Periodontitis, a marker of risk in pregnancy for preterm birth. J Clin Periodontol. 2005 Jan;32(1):45–52.

83. Katz J, Chegini N, Shiverick KT, Lamont RJ. Localization of P. gingivalis in preterm delivery placenta. J Dent Res. 2009 Jun;88(6):575–8.

84. Riviere GR, Riviere KH, Smith KS. Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. Oral Microbiol Immunol. 2002 Apr;17(2):113–8.

85. Poole S, Singhrao SK, Kesavalu L, Curtis MA, Crean S. Determining the presence of periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. J Alzheimers Dis.

2013;36(4):665–77.

86. Kamer AR, Craig RG, Pirraglia E, Dasanayake AP, Norman RG, Boylan RJ, Nehorayoff A, Glodzik L, Brys M de LM. TNF-alpha and antibodies to periodontal bacteria discriminate between Alzheimer's disease patients and normal subjects. J Neuroimmunol. 2009;30(216):97–97.

87. Sparks Stein P, Steffen MJ, Smith C, Jicha G, Ebersole JL, Abner E DD 3rd. Serum antibodies to periodontal pathogens are a risk factor for Alzheimer's disease. Alzheimers Dement . 2012;8(3):196–203.

88. Noble JM, Borrell LN, Papapanou PN, Elkind MS V, Scarmeas N, Wright CB. Periodontitis is associated with cognitive impairment among older adults: analysis of NHANES-III. J Neurol Neurosurg Psychiatry. 2009 Nov;80(11):1206–11.

89. Hajishengallis G. The inflammophilic character of the periodontitis-associated microbiota. Mol Oral Microbiol. 2014 Dec;29(6):248–57.

90. Kocgozlu L, Elkaim R, Tenenbaum H, Werner S. Variable cell responses to P. gingivalis lipopolysaccharide. J Dent Res. 2009 Aug;88(8):741–5.

91. Olsen I, Singhrao SK. Can oral infection be a risk factor for Alzheimer's disease? J Oral Microbiol. 2015;7(1):1–16.

92. Offenbacher S, Beck JD, Moss K, Mendoza L, Paquette DW, Barrow DA, et al. Results from the Periodontitis and Vascular Events (PAVE) Study: a pilot multicentered, randomized, controlled trial to study effects of periodontal therapy in a secondary prevention model of cardiovascular disease. J Periodontol. 2009 Feb;80(2):190–201.

93. Desvarieux M, Demmer RT, Jacobs DR, Papapanou PN, Sacco RL, Rundek T. Changes in clinical and microbiological periodontal profiles relate to progression of carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology study. J Am Heart Assoc. 2013;2(6):e000254.

94. Madianos PN, Bobetsis YA, Offenbacher S. Adverse pregnancy outcomes (APOs) and periodontal disease: pathogenic mechanisms. J Periodontol. 2013 Apr;84(4 Suppl):S170–80.

95. Han YW, Fardini Y, Chen C, Iacampo KG, Peraino VA, Shamonki JM, et al. Term stillbirth caused by oral Fusobacterium nucleatum. Obstet Gynecol. NIH Public Access; 2010 Feb;115(2 Pt 2):442–5.

96. Sanz M, Kornman K. Periodontitis and adverse pregnancy outcomes: Consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. J Clin Periodontol. 2013;40(SUPPL. 14):164–9.

97. Scher JU, Bretz WA, Abramson SB. Periodontal disease and subgingival microbiota as contributors for rheumatoid arthritis pathogenesis: modifiable risk factors? Curr Opin Rheumatol. 2014 Jul;26(4):424–9.

98. Joseph R, Rajappan S, Nath SG, Paul BJ. Association between chronic periodontitis and rheumatoid arthritis: a hospital-based case-control study. Rheumatol Int. 2013 Jan;33(1):103–9.

99. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. Nature Publishing Group; 2012 Sep 26;490(7418):55–60.

100. Miklossy J. Emerging roles of pathogens in Alzheimer disease. Expert Rev Mol Med. 2011;13:e30.

101. Green PS, Mendez AJ, Jacob JS, Crowley JR, Growdon W, Hyman BT, et al. Neuronal expression of myeloperoxidase is increased in Alzheimer's disease. J Neurochem. 2004 Aug;90(3):724–33.

102. Tarkowski E, Blennow K, Wallin A, Tarkowski A. Intracerebral production of tumor necrosis factor-alpha, a local neuroprotective agent, in Alzheimer disease and vascular dementia. J Clin Immunol. 1999 Jul;19(4):223–30.

103. Killick R, Hughes TR, Morgan BP, Lovestone S. Deletion of Crry, the murine ortholog of the sporadic Alzheimer's disease risk gene CR1, impacts tau phosphorylation and brain CFH. Neurosci Lett. Elsevier; 2013 Jan 15;533:96–9.

104. Matas SLDA, Glehn F Von, Fernandes GBP, Soares CAS. Cerebrospinal fluid analysis in the context of CNS demyelinating diseases. Arq Neuropsiquiatr. 2013 Sep;71(9B):685–8.

105. Leal MC, Casabona JC, Puntel M, Pitossi FJ. Interleukin-1β and tumor necrosis factor-α: reliable targets for protective therapies in Parkinson's Disease? Front Cell Neurosci. Frontiers Media SA; 2013;7:53.

106. Nagatsu T, Mogi M, Ichinose H, Togari A. Changes in cytokines and neurotrophins in Parkinson's disease. J Neural Transm Suppl. 2000;(60):277–90.

107. Holmes C, Cunningham C, Zotova E, Woolford J, Dean C, Kerr S, et al. Systemic inflammation and disease progression in Alzheimer disease. Neurology. 2009 Sep 8;73(10):768–74.

108. Dunn N, Mullee M, Perry VH, Holmes C. Association between dementia and infectious disease: evidence from a case-control study. Alzheimer Dis Assoc Disord. 19(2):91–4.

109. Miklossy J, Marshall B, Warren J, Laitinen K, Laurila A, Pyhälä L, et al. Alzheimer's disease - a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. J Neuroinflammation. BioMed Central; 2011;8(1):90.

110. Snowdon DA, Greiner LH, Mortimer JA, Riley KP, Greiner PA, Markesbery WR. Brain infarction and the clinical expression of Alzheimer disease. The Nun Study. JAMA. 1997 Mar 12;277(10):813–7.

111. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol. Nature Publishing Group; 2014;15(1):30–44.

112. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, McIntosh ML, Alsam A, Kirkwood KL, Lambris JD, Darveau RP CM. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe. 2011;10(5):497–506.

113. Maekawa T, Krauss JL, Abe T, Jotwani R, Triantafilou M, Triantafilou K, Hashim A, Hoch S, Curtis MA, Nussbaum G, Lambris JD HG. Porphyromonas gingivalis manipulates complement and TLR signaling to uncouple bacterial clearance from inflammation and promote dysbiosis. Cell Host Microbe. 2014;15(6):768–78.

114. Jiao Y, Darzi Y, Tawaratsumida K, Marchesan JT, Hasegawa M, Moon H, et al. Induction of bone loss by pathobiont-mediated Nod1 signaling in the oral cavity. Cell Host Microbe. 2013 May 15;13(5):595–601.

115. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol. 2012 Dec;27(6):409–19.

116. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. Nat Rev Endocrinol. 2011 Dec;7(12):738–48.

117. Lundberg K, Wegner N, Yucel-Lindberg T, Venables PJ. Periodontitis in RA-the citrullinated enolase connection. Nat Rev Rheumatol. 2010 Dec;6(12):727–30.

118. Genco RJ, Van Dyke TE. Prevention: Reducing the risk of CVD in patients with periodontitis. Nat Rev Cardiol. 2010 Sep;7(9):479–80.

119. Bartosch S, Fite A, Macfarlane GT, Mcmurdo MET. Characterization of Bacterial Communities in Feces from Healthy Elderly Volunteers and Hospitalized Elderly Patients by Using Real-Time PCR and Effects of Antibiotic Treatment on the Fecal Microbiota Characterization of Bacterial Communities in Feces from. Appl Environ Microbiol. 2004;70(6):3575–81.

120. Ufnar JA, Wang SY, Christiansen JM, Yampara-Iquise H, Carson CA, Ellender RD. Detection of the nifH gene of Methanobrevibacter smithii: A potential tool to identify sewage pollution in recreational waters. J Appl Microbiol. 2006;101(1):44–52.

121. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes, application to the identification of mycorrihiza and rusts. Mol Ecol. 1993;2:113–8.

122. Aminov RI, Mackie RI. Molecular Ecology of Tetracycline Resistance : Development and Validation of Primers for Detection of Tetracycline Resistance Genes Encoding Ribosomal Protection Proteins Molecular Ecology of Tetracycline Resistance : Development and Validation of Primers. Appl Environ Microbiol. 2001;67(1):22–3.

123. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol. 2002;68(11):5445–51.

124. Rinne MM, Gueimonde M, Kalliomäki M, Hoppu U, Salminen SJ, Isolauri E. Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. FEMS Immunol Med Microbiol. 2005;43(1):59–65.

125. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in

human feces. Appl Environ Microbiol. 2002 Nov;68(11):5445–51.

126. Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 2004;97(6):1166–77.

127. Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, de Vos WM. Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol. 2002 Jan;68(1):114–23.

128. Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, Ouellette M, et al. Development of a PCR Assay for Identification of Staphylococci at Genus and Species Levels. J Clin Microbiol. American Society for Microbiology; 2001 Jul;39(7):2541–7.

129. Collado MC, Delgado S, Maldonado A, Rodr??guez JM. Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. Lett Appl Microbiol. 2009;48(5):523–8.

130. Collado MC, Derrien M, Isolauri E, De Vos WM, Salminen S. Intestinal integrity and Akkermansia muciniphila, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol. 2007;73(23):7767–70.

131. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183–9.

132. Achenbach L WC. 16S and 23S rRNA-like primers. In: Sower K, Schreier H, editors. Archaea: A Laboratory Manual. New York, USA: Cold Spring Harbor Laboratory Press; 1995. p. 521–3.

133. Kušar D, Avguštin G. Molecular profiling and identification of methanogenic archaeal species from rabbit caecum. FEMS Microbiol Ecol. 2010;74(3):623–30.

134. Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 1993 Mar;59(3):695–700.

135. White TJ, Bruns T, Lee S TJ. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T, editors. PCR Protocols: a guide to methods and applications. New York, USA: Academic Press; 1990. p. 315–22.

136. Duong LM, Jeewon R, Lumyong S, Hyde KD. DGGE coupled with ribosomal DNA gene phylogenies reveal uncharacterized fungal phylotypes.

137. Schmieder Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics 2011; 27(6): 863-4.

138. Erik Aronesty (2011). ea-utils: Command-line tools for processing biological sequencing data; http://code.google.com/p/ea-utils.

139. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335–6.

140. García-Ptacek S, Faxén-Irving G, Cermáková P, Eriksdotter M, Religa D. Body mass index in dementia. Eur J Clin Nutr. 2014 July:1–6.

141. Uttara B, Singh A V, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol. Bentham Science Publishers; 2009 Mar;7(1):65–74.

142. Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R. Atherosclerosis and oxidative stress. Histol Histopathol. 2008 Mar;23(3):381–90.

143. Tzikas S, Schlak D, Sopova K, Gatsiou A, Stakos D, Stamatelopoulos K, et al. Increased myeloperoxidase plasma levels in patients with Alzheimer's disease. J Alzheimers Dis. 2014;39(3):557–64.

144. Popp J, Wolfsgruber S, Heuser I, Peters O, Hüll M, Schröder J, et al. Cerebrospinal fluid cortisol and clinical disease progression in MCI and dementia of Alzheimer's type. Neurobiol Aging. Elsevier Inc; 2015;36(2):601–7.

145. Gomes BPFA, Jacinto RC, Pinheiro ET, Sousa ELR, Zaia AA, Ferraz CCR, et al. Porphyromonas gingivalis,

Porphyromonas endodontalis, Prevotella intermedia and Prevotella nigrescens in endodontic lesions detected by culture and by PCR. Oral Microbiol Immunol. 2005 Aug;20(4):211–5.

146. Lehallier B, Essioux L, Gayan J, Alexandridis R, Nikolcheva T, Wyss-Coray T, et al. Combined Plasma and Cerebrospinal Fluid Signature for the Prediction of Midterm Progression From Mild Cognitive Impairment to Alzheimer Disease. JAMA Neurol. 2015 Dec 14;1–10.

147. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. BMJ Group; 2006 Feb;55(2):205–11.

148. Ott SJ, Schreiber S. Reduced microbial diversity in inflammatory bowel diseases. Gut. BMJ Group; 2006 Aug;55(8):1207.

149. Sun J, Li Y. Reduce oral microbial diversity in individuals habour periodontal diseases. Dent Hypotheses. 2012;3(1):16–22.

150. Ling Z, Liu X, Cheng Y, Jiang X, Jiang H, Wang Y, et al. Decreased Diversity of the Oral Microbiota of Patients with Hepatitis B Virus-Induced Chronic Liver Disease: A Pilot Project. Sci Rep. Nature Publishing Group; 2015 Nov; 26(5)17098.

151. Maukonen J, Mättö J, Suihko M-L, Saarela M. Intra-individual diversity and similarity of salivary and faecal microbiota. J Med Microbiol. 2008 Dec; 57(12):1560–8.

152. Dewhirst FE, Klein EA, Thompson EC, Blanton JM, Chen T, Milella L, et al. The Canine Oral Microbiome. Ravel J, editor. PLoS One. Public Library of Science; 2012 Apr 27;7(4):e36067.

153. Scully C, el-Kabir M, Samaranayake LP. Candida and oral candidosis: a review. Crit Rev Oral Biol Med. 1994;5(2):125–57.

154. de Steenhuijsen Piters W a a, Huijskens EGW, Wyllie AL, Biesbroek G, van den Bergh MR, Veenhoven RH, et al. Dysbiosis of upper respiratory tract microbiota in elderly pneumonia patients. ISME J. 2015;1–12.

155. Fukui K, Kato N, Kato H, Watanabe K, Tatematsu N. Incidence of Prevotella intermedia and Prevotella nigrescens carriage among family members with subclinical periodontal disease. J Clin Microbiol. American Society for Microbiology (ASM); 1999 Oct;37(10):3141–5.

156. Ardila Medina CM, Alzate Vega J, Guzmán Zuluaga IC. Asociación de Prevotella intermedia/nigrescens, bacilos entéricos gram-negativos y parámetros clínicos en periodontitis crónica. Av en Periodoncia e Implantol Oral. Ediciones Avances, S.L.; 2013;25(3):165–70.

157. Medikeri RS, Lele SV, Jain PM, Mali P, Medikeri MR. Quantification of selenomonas sputigena in chronic periodontitis in smokers using 16S rDNA based PCR analysis. J Clin Diagnostic Res. 2015;9(4):ZC13–7.

158. Gonçalves LFH, Fermiano D, Feres M, Figueiredo LC, Teles RP, Mayer MPA, et al. Levels of Selenomonas species in generalized aggressive periodontits. 2013;47(6):711–8.

159. Slenker AK, Hess BD, Jungkind DL, DeSimone JA. Fatal Case of Weeksella virosa Sepsis. J Clin Microbiol. American Society for Microbiology; 2012 Dec ;50(12):4166–7.

160. Davé V, Street K, Francis S, Bradman A, Riley L, Eskenazi B, et al. Bacterial microbiome of breast milk and child saliva from low-income Mexican-American women and children. Pediatr Res. 2016.

161. Eribe ERK, Olsen I. Leptotrichia species in human infections. Anaerobe. 2008;14(3):131–7.

162. Coit P, Mumcu G, Ture-Ozdemir F, Unal AU, Alpar U, Bostanci N, et al. Sequencing of 16S rRNA reveals a distinct salivary microbiome signature in Behçet's disease. Clin Immunol. Elsevier Inc.; 2016;169:28–35.

163. Guerrero-Preston R, Godoy-Vitorino A, Jedlicka A, Rodriguez H, Gonzalez H, Sidransky D. 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, Human Papilloma Virus infection and surgical treatment. Oncotarget. 2016;1–15.

164. Nelson DB, Hanlon AL, Wu G, Liu C, Fredricks DN. First Trimester Levels of BV-Associated Bacteria and Risk of Miscarriage Among Women Early in Pregnancy. Matern Child Health J. Springer US; 2015;19(12):2682–7.

165. Tai M-LS, Velayuthan RD. Sphingomonas paucimobilis: an unusual cause of meningitis-case report. Neurol Med Chir (Tokyo). 2014;54(4):337–40.

166. Hajiroussou V, Holmes B, Bullas J, Pinning CA. Meningitis caused by Pseudomonas paucimobilis. J Clin Pathol. BMJ Group; 1979 Sep;32(9):953–5.

167. Hsueh PR, Teng LJ, Yang PC, Chen YC, Pan HJ, Ho SW, et al. Nosocomial infections caused by Sphingomonas paucimobilis: clinical features and microbiological characteristics. Clin Infect Dis. 1998 Mar;26(3):676–81.

168. Toh H, Tay H, Kuar W, Weng T, Tang H, Tan C. Risk factors associated with Sphingomonas paucimobilis infection. J Microbiol Immunol Infect. 2011;44(4):289–95.

169. Mohammed J, Mattner J. Autoinmune disease triggered by infection with alphaproteobacteria. Expert REv Clin Immunol. 2010;48(Suppl 2):1–6.

170. Mikx FH, van der Hoeven JS, König KG, Plasschaert AJ, Guggenheim B. Establishment of defined microbial ecosystems in germ-free rats. I. The effect of the interactions of streptococcus mutans or Streptococcus sanguis with Veillonella alcalescens on plaque formation and caries activity. Caries Res. 1972;6(3):211–23.

171. Arif N, Sheehy EC, Do T, Beighton D. Diversity of Veillonella spp. from sound and carious sites in children. J Dent Res. Europe PMC Funders; 2008 Mar;87(3):278–82.

172. Camelo-castillo AJ, Mira A, Pico A, Nibali L, Henderson B, Donos N, et al. Subgingival microbiota in health compared to periodontitis and the influence of smoking. Front Microbiol. 2015;6(Feb):1–12.

173. Brinig MM, Lepp PW, Ouverney CC, Armitage GC, Relman DA. Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. Appl Environ Microbiol. 2003;69(3):1687–94.

174. Kuehbacher T, Rehman A, Lepage P, Hellmig S, F??lsch UR, Schreiber S, et al. Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. J Med Microbiol. 2008;57(12):1569–76.

SUPLEMENTARY DATA

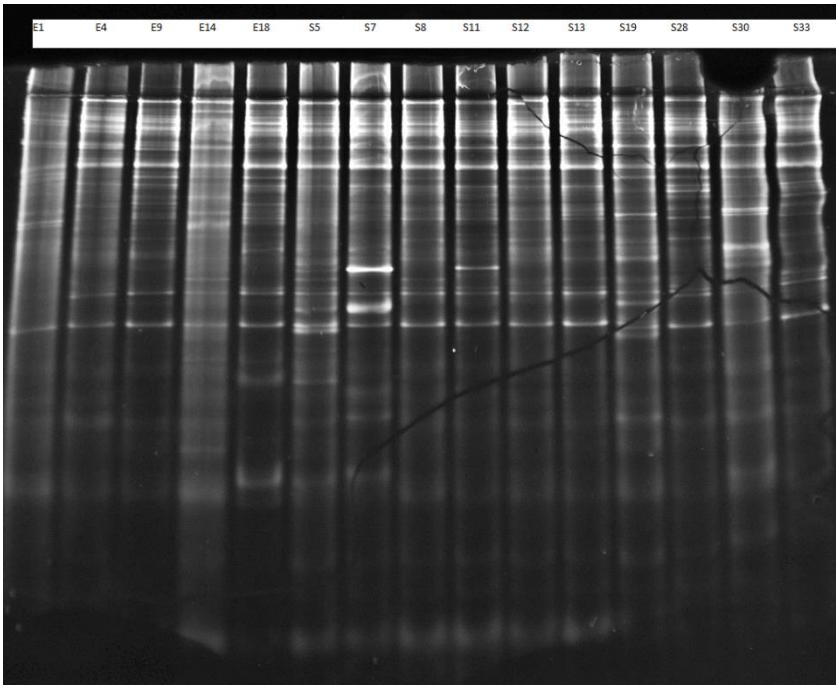

Figure 1. DGGE of archaea species in NCF and CCF individuals

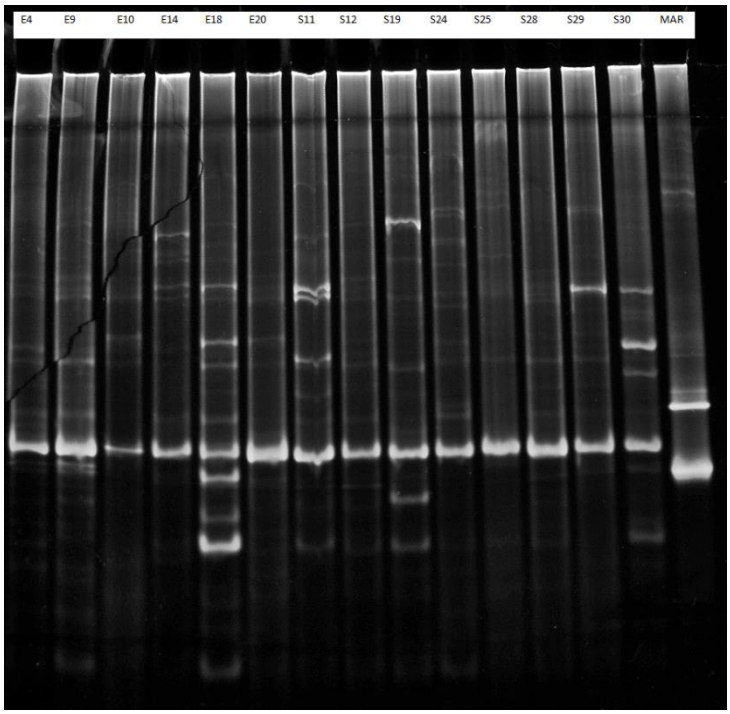

Figure 2. DGGE from fungal species in CCF and NCF individuals

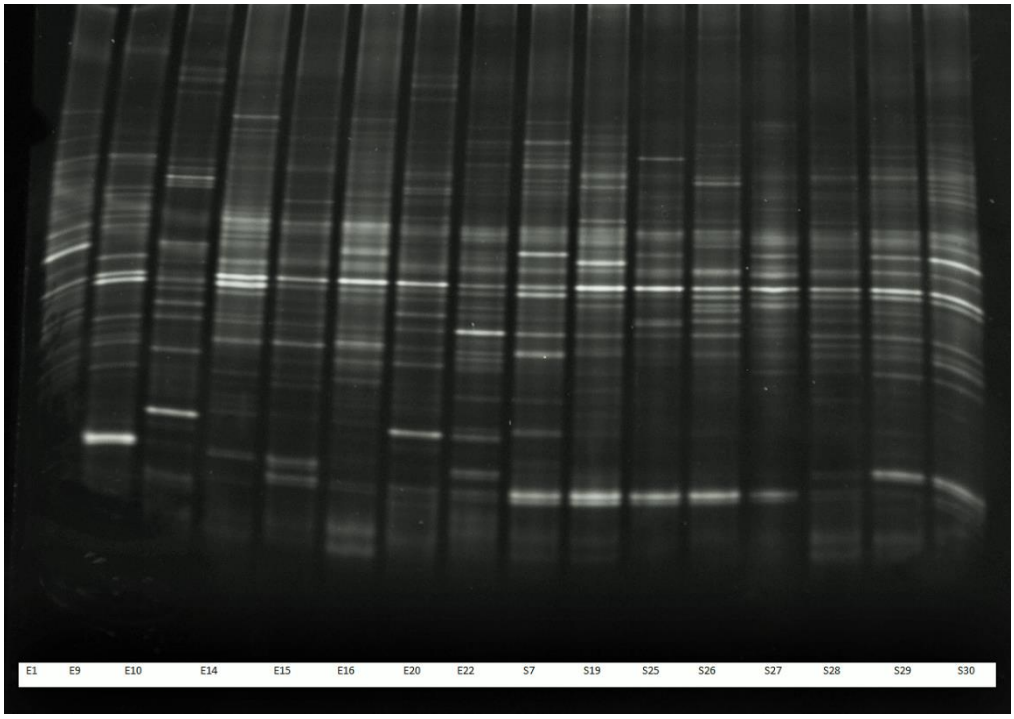

Figure 3. DGGE of bacterial species presents in NCF and CCF individuals.

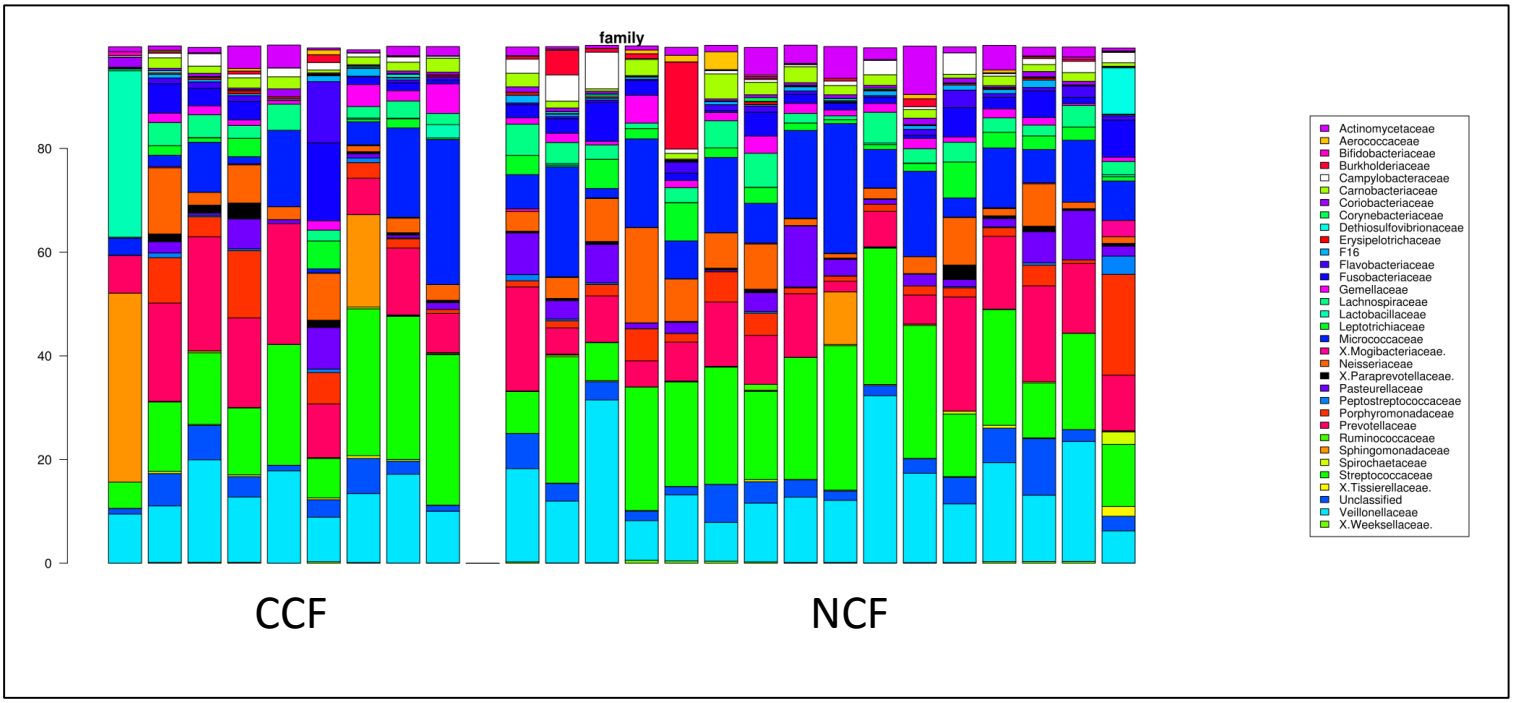

Figure 4. Bar charts of the composition of microbiota at the family level in the NCF and CCF individuals

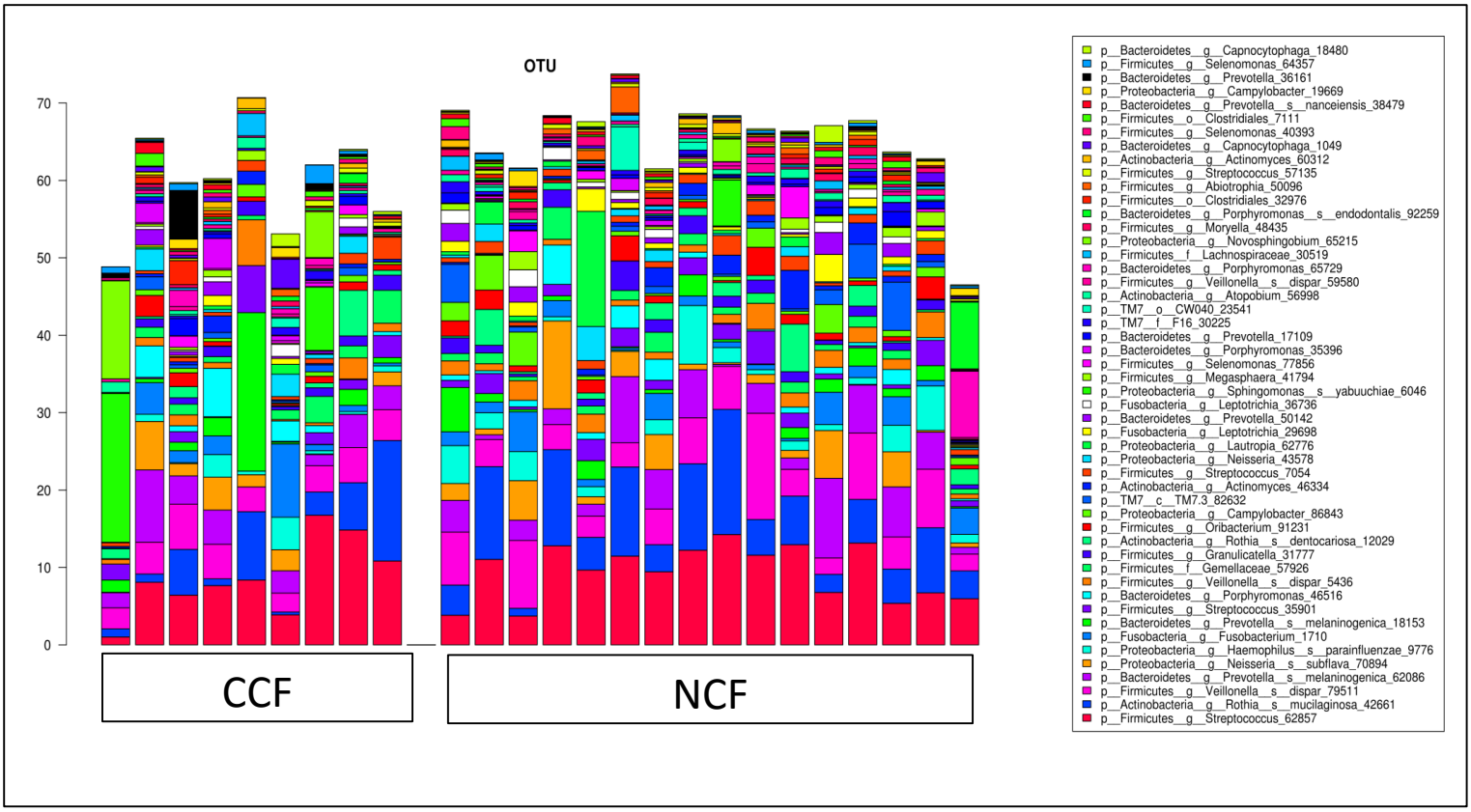

Figure 5. Bar charts of the OTUs of the microbiota present in the NCF and CCF individuals

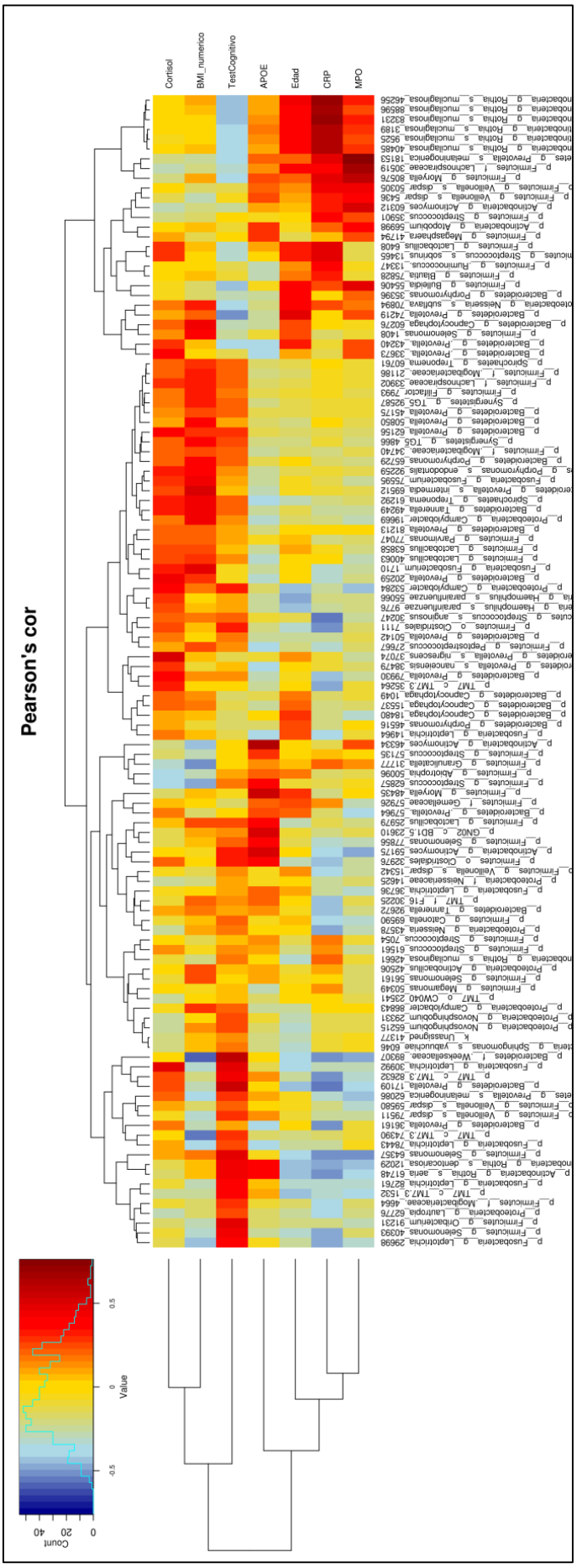

Figure 6. HeatMap of the relationship of some microbiota OTUs and the parameters measured in the study.

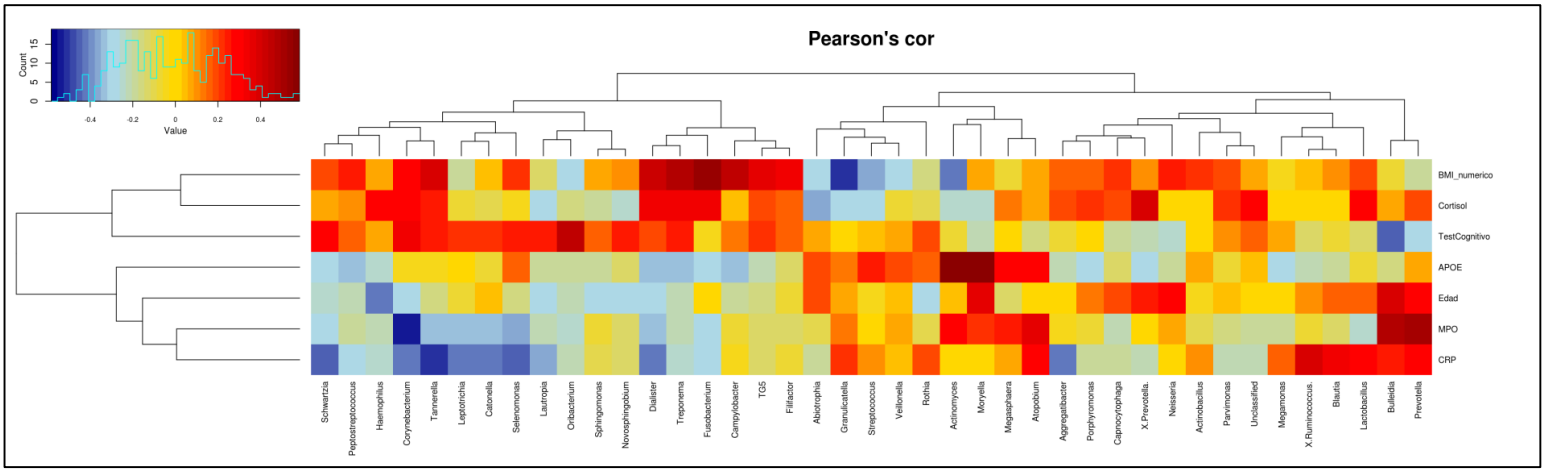

Figure 7. HeatMap ot the relationship between microbiota at level of genus and the parameters measured in the studio.

Table 1. Relationship between specific OTUs and cortisol levels

Taxa	P-value	R	Mean Abundance	Positive Samples
p__Bacteroidetes__g__Prevotella__s__nigrescens_37074	0.0047	0.5566	0.402	23
p__Bacteroidetes__g__Prevotella_20259	0.0140	0.4944	0.434	24
p__TM7__c__TM7.3_35264	0.0290	0.4456	0.397	23
p__Firmicutes__g__Selenomonas_64357	0.0380	0.4265	0.474	23
p__Firmicutes__f__Lachnospiraceae_30519	0.0930	-0.3503	0.586	23

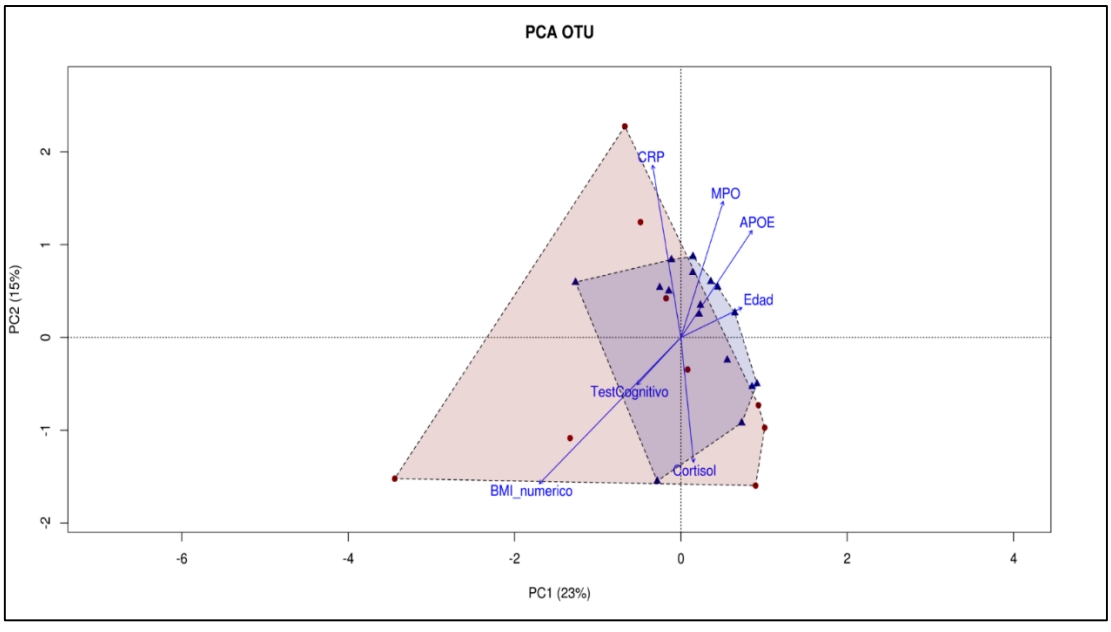

Figure 8. PCA and bi-plot of the NCF (red) and CCF (blue) and the other parameters measured.