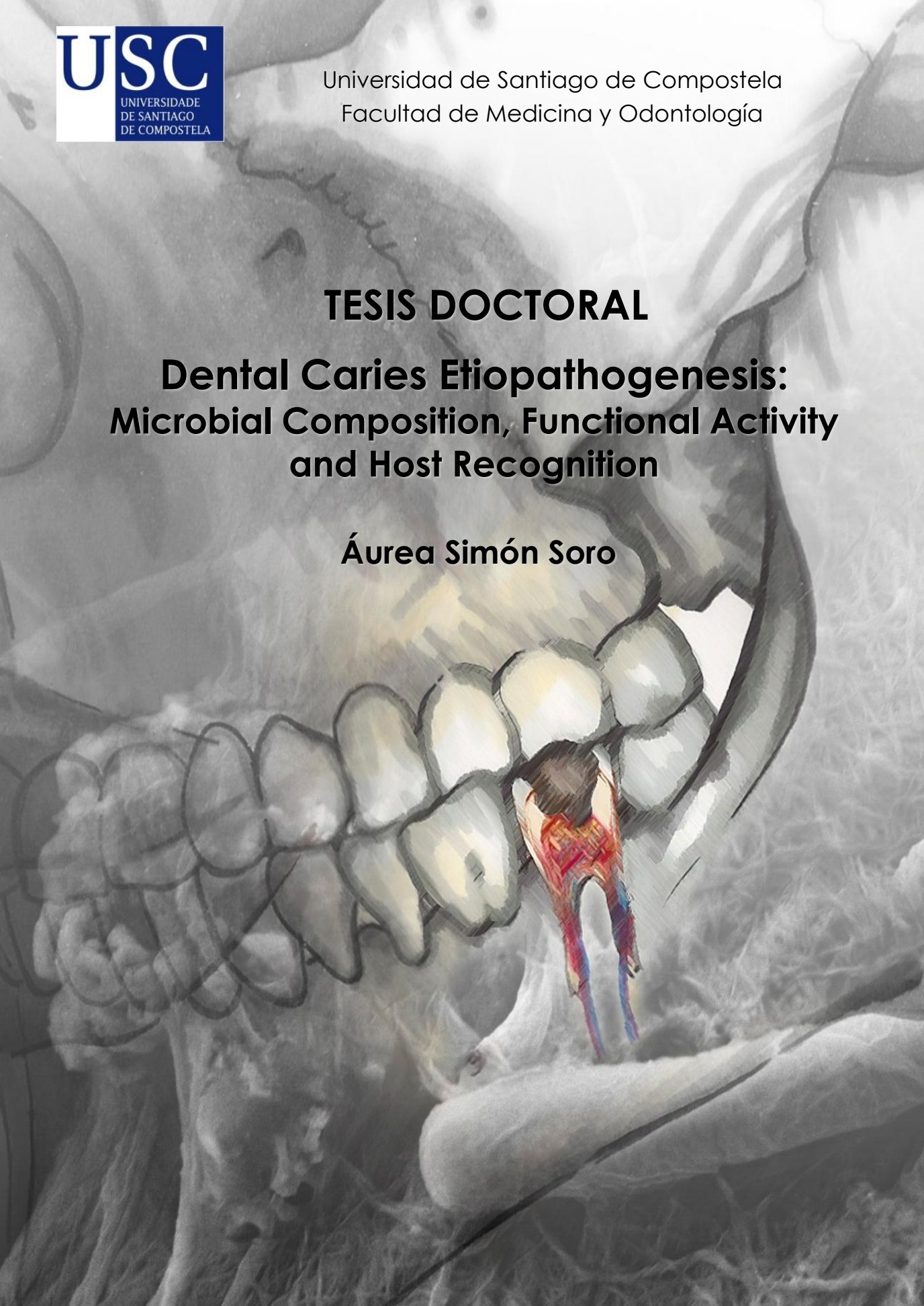
Universidad de Santiago de Compostela
Facultad de Medicina y Odontología

TESIS DOCTORAL

Dental Caries Etiopathogenesis:
Microbial Composition, Functional Activity and Host Recognition

Áurea Simón Soro



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Caries Etiopathogenesis: Microbial Composition, Functional Activity and Host Recognition

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El **Dr. Alejandro Mira Obrador**, investigador titular de la Fundación para el fomento de la investigación sanitaria y biomédica de la Comunidad Valenciana.

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HACEN CONSTAR:

Como directores de la Tesis Doctoral que lleva por título "***Etiopatogénesis de la Caries Dental: Composición Microbiana, Actividad Funcional e Interacción con el Hospedador***", realizada por la licenciada en Odontología Dña. Áurea Simón Soro, que cumple todos los requisitos para ser presentada y defendida ante el oportuno tribunal para optar al Grado de Doctor en Odontología por la Universidad de Santiago de Compostela.

Dr. A. Mira Obrador

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AGRADECIMIENTOS

Creer en ti mismo, no depende únicamente de uno, sino es la suma de muchas manos que se han ido entrelazando para marcar el camino.

En esta andadura he ido encontrando personas maravillosas que me han mostrado que nada está escrito. Si fuera por mí, muchas veces hubiera optado por el trayecto sin pendiente, a veces incluso circular, caminando sin rumbo.

Pero la meta es la que es. Desde el lucero del alba quien me ha guiado cada día hasta los peregrinos que han aparecido enriqueciendo mi andadura, me han ayudado a continuar.

Este agradecimiento va para todos ellos, las luces que marcan mi destino. Porque siempre ha sido, es y será por el arte de la ciencia.

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Thesis Summary

"Dental Caries Etiopathogenesis: Microbial Composition, Functional Activity and Host Recognition"

For decades, the sugar-fermenting, acidogenic species *Streptococcus mutans* has been considered the main causative agent of dental caries and most diagnostic and therapeutic strategies have been targeted toward this microorganism. However, the DNA- and RNA-based studies from carious lesions reported in this thesis, have uncovered an extraordinarily diverse ecosystem where *S. mutans* accounts only for a tiny fraction of the bacterial community. This supports the concept that consortia formed by multiple microorganisms act collectively, probably synergistically, to initiate and expand the carious lesion. The data also show that these microbial consortia are different between individuals, between the affected tissue, and even between different lesions from the same individual. Thus, antimicrobial therapies are not expected to be effective in the treatment of caries and other polymicrobial diseases that do not follow Koch's postulates, and that I propose cannot be considered infectious diseases in classical terms. In addition, the data also indicate a prominent role for the immune system in caries risk, suggesting that therapies directed towards stimulating immunological competence should be explored. Based on the results from this Thesis, I propose that dental caries is a dysbiotic polymicrobial disease caused by pathobionts.

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General Introduction

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I.1. WHAT IS DENTAL CARIES?

Dental caries is the result – the signs and symptoms – of a localized chemical dissolution of the tooth surface caused by metabolic events of microorganisms taking place in the dental plaque covering the affected area. The destruction can affect enamel, dentin and/or cementum (Fejerskov O, 2008). Enamel is the hardest and most highly mineralized substance of the body. Ninety-six percent of enamel consists of the mineral hydroxyapatite, with water and organic material composing the rest. Dentin is secreted by the odontoblasts of the dental pulp and is a mineralized connective tissue made up of 70% inorganic materials, 20% organic materials, and 10% water. Cementum is a specialized bony substance covering the root of a tooth and is approximately 45% inorganic material (mainly hydroxyapatite), 33% organic material (mainly collagen) and 22% water.

In the oral cavity, there is a constant exchange of metabolites and ions such as calcium and phosphate between the mineral surface of the teeth and the salivary compounds (Figure I.1.a). In normal conditions the processes of demineralization and remineralization are balanced. However, when the pH derived from the metabolism of microorganisms which are part of dental biofilm is lower than a certain threshold, normally established around 5.5 (ten Cate JM, 2009), mineral loss is higher than restructuring. This point is a microstructural start-point for dental caries.

The signs and symptoms associated with the disease initiation and progression depend directly on the depth of loss of mineral and the involvement of another vital tissue such as dentin, which has sensitivity to different stimuli (Figure I.1.b).

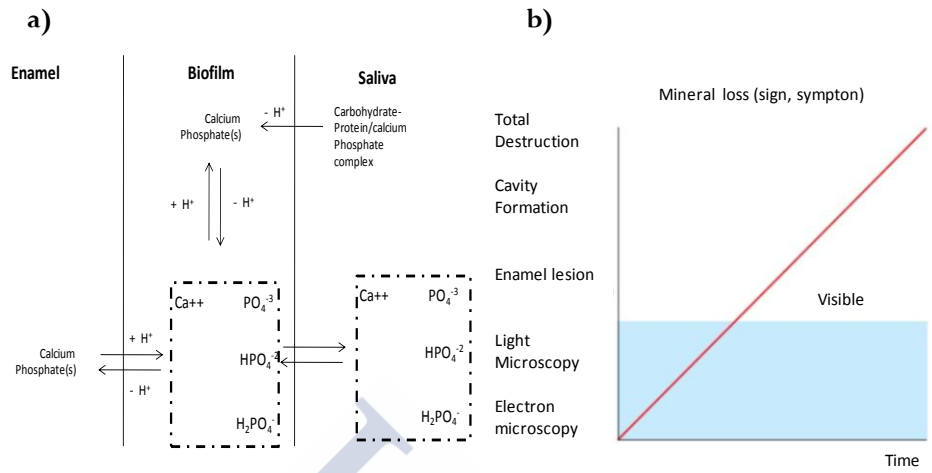


Figura I.1. Chemical and mineral development of dental caries. a) Scheme of the re- and demineralization process of teeth indicating metabolites exchange. b) Principal progress of mineral loss in relation to time. The slope of the line may vary depending on the caries challenge, and time may range from weeks to months and years (Kidd EA and Fejerskov O, 2008).

Types of caries can be classified according to the affected surface, such as coronal or root caries and to whether the affected tooth is permanent or deciduous. In the current Thesis, all samples have been collected from adult individuals and therefore only permanent teeth have been studied. Topographically, caries are interproximal, occlusal, incisal, buccal or palatine, all these being considered as coronal caries. Also, the classification depends on the damaged tissue, namely enamel, dentin or pulp. Black's categorization allowed to better differentiate caries by combining the affected tooth and surface in his classification system (Fisher J et al., 2012). Caries lesions reflect the activity level in the biofilm (Kidd EA and Fejerskov O, 2004), which is responsible for the initiation and progression of the disease. Thus, another important characteristic to classify caries relates to its activity, and lesions have been classified as active or arrested according to their texture, colour and brightness (Nyvad B and Fejerskov O, 1997; Nyvad B et al., 1999, Nyvad B et al., 2003).

I.2. HISTOPATHOLOGY OF DENTAL CARIES

In the current Thesis, in order to study histopathologically well-defined caries lesions and to describe the microbiology associated with different stages of the disease, I focused on enamel and dentin caries processes. Thus, I have not included root caries lesions and the microbiology associated with cement lesions has not been considered.

I.2.1. Enamel Stage: Ultrastructural and White Spot Lesions

The initial stage of dental caries starts as a not visible or ultrastructural lesion of enamel, as a result of the biofilm activity. Three stages have been described as directly associated with microscopic structural changes of enamel mineral composition. After a week of experimental results, Holmen et al. (1985) found signs of direct dissolution of the outer enamel surface, where an enlargement of the intercrystalline spaces due to partial dissolution of the individual crystal peripheries was seen. At the second week of the same experiment, these authors observed a complete dissolution of the thin perikymata overlappings; a marked dissolution corresponding to developmental irregularities such as pits and focal holes; and continued enlargement of the intercrystalline spaces. The direct surface erosion is most likely partly responsible for the matte surface of the active lesion.

The so-called white spot lesions correspond to the first visible stage of dental caries and have a chalky white appearance. Their development has been studied in teeth that were extracted for orthodontic reasons, after detailed examination of sections in polarized light (Holmen et al., 1985b). After only one week of biofilm formation, this examination showed a slight increase in enamel

porosity, especially in the tissue beneath the outer microsurface. This so-called subsurface demineralization became more obvious after several weeks, giving rise to two clear histological zones (Silverstone LM, 1973). Apart from fluoride, a protective role of salivary proline-rich proteins and other salivary inhibitors, such as statherin, has been demonstrated (Hay DI, 1984). These inhibit demineralization and prevent crystal growth. They are macromolecules and cannot penetrate into the deeper parts of the enamel; thus, their stabilizing role is limited to the enamel surface. White spot lesions are non-cavitated and can advance to a cavitated lesion that can be clinically detected. Cavitation usually occurs because of external forces that eventually lead to the collapse of the outer surface, which in turn leads to a discontinuity or break in the surface. This stage of the disease is not reversible and generally requires operative intervention to restore function and to arrest the caries process.

I.2.2. Dentin Stage: Tubular Sclerosis and Reactionary Dentin

Dentin is a vital tissue containing the odontoblast processes, and this tissue will react to transmission of stimuli from the oral cavity through the microporous enamel (Bleicher F, 2014). When the caries lesion reaches dentin, the most common defense reaction by the pulp-dentin complex is the deposition of mineral within the dentinal tubules. This is called ‘tubular sclerosis’ or ‘translucent dentin’ because, due to reduced light scattering, the tissue appears histologically translucent when it is examined by transmitted light microscopy. The process requires a vital odontoblast and can also be activated by physical injury like bruxism or by age.

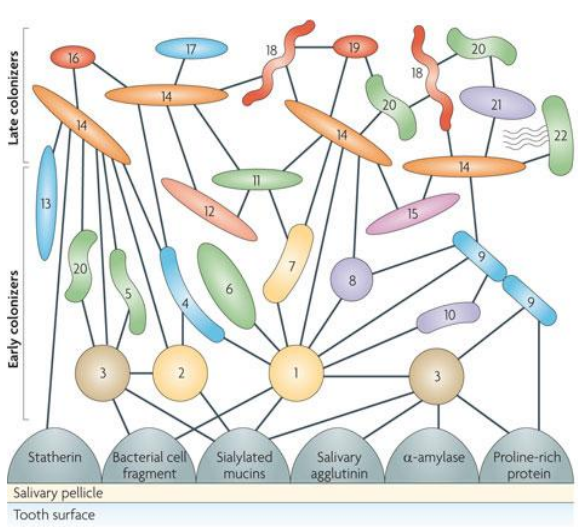
Tubular sclerosis in dentin is visible before the enamel lesion extends to the enamel-dentin junction. The other important defense mechanism of the

pulp-dentin complex is the formation of reactionary dentin, which may be initiated before bacterial invasion of the dentin. Deposition of reactionary dentine by surviving odontoblast cells can arise at the pulp-dentine interface and at a peritubular/intratubular location in response to an appropriate stimulus. Some of this reactionary dentine may be a feature of normal physiological processes, such as aging, but the majority of reactionary dentine is considered to arise in response to dental disease (Goldberg et al., 2011).

I.3. MICROBIOLOGY OF DENTAL CARIES

I.3.1. Dental Plaque as a Biofilm

Dental biofilm is 3-D accumulation of interacting microorganisms attached to a tooth surface, embedded in a matrix of extracellular polymers (Marsh PD, 2010). The biofilm is developed by a succession of microorganisms (Kolenbrander PE et al., 2010), which are classified according to their involvement at the time of formation in initial, early, middle and late colonizers (Figure I.2).



1. *Streptococcus oralis* and *S. sanguinis*
2. *Streptococcus mitis*
3. *Streptococcus gordonii*
4. *Capnocytophaga ochracea*
5. *Propionibacterium acnes*
6. *Haemophilus parainfluenzae*
7. *Prevotella loescheii*
8. *Veillonella* spp.
9. *Actinomyces oris* and *A. naeslundii*
10. *Eikenella corrodens*
11. *Actinomyces israelii*
12. *Capnocytophaga gingivalis*
13. *Capnocytophaga sputigena*
14. *Fusobacterium nucleatum*
15. *Prevotella denticola*
16. *Aggregatibacter actinomycetemcomitans*
17. *Eubacterium* spp.
18. *Treponema denticola*
19. *Tannerella forsythia*
20. *Porphyromonas gingivalis*
21. *Prevotella intermedia*
22. *Selenomonas flueggei*

Figure I.2. Oral biofilm model proposed by Kolenbrander et al. (2010). Early colonizers (framed in green) bind to the salivary pellicle on the tooth surface and coaggregate with other bacterial species in a sequencial manner. *Fusobacterium* appears to have multiple interactions and it has been proposed as a "bridge" between early and late colonizers (framed in blue), which are mainly anaerobic and include several pathogenic organisms (Kolenbrander PE et al., 2010).

The full development of a supragingival biofilm containing all compounds which shape the dental plaque, such as glucopolysaccharides that allowing the network of bacteria to attach to each other and these with the tooth surface, is a process that involves 24 hours.

The first step in biofilm development is the formation of the acquired pellicle (Hannig C and Hannig M, 2009). It is a combination of active proteins and glycoproteins from saliva and gingival crevicular fluid, which attach to the tooth surface seconds after toothbrushing or eruption (Marsh PD, 2010). The complex comprises statherins, mucins, agglutinins, alpha-amylase and prolin-rich proteins. They act like chains between tooth and early colonizers to begin the oral biofilm formation. Only in 4 hours, mainly aerobic species of the genera

Streptococcus, *Capnocytophaga*, *Veillonella* or *Actinomyces* (known of as initial and early colonizers) adhere to the tooth proteic film through adhesins, forming a first layer of biofilm.

A second layer of biofilm develops when other microorganisms are attached to first colonizers, through adhesion of their respective cell surfaces (Kolenbrander PE et al., 2010); this mechanism is known as coaggregation or coadhesion. In this step, the biofilm becomes mainly anaerobic. Middle colonizers, such as *Fusobacterium nucleatum*, and late colonizers, like *Lactobacillus* spp., contribute to the second layer formation (Figure I.3.C).

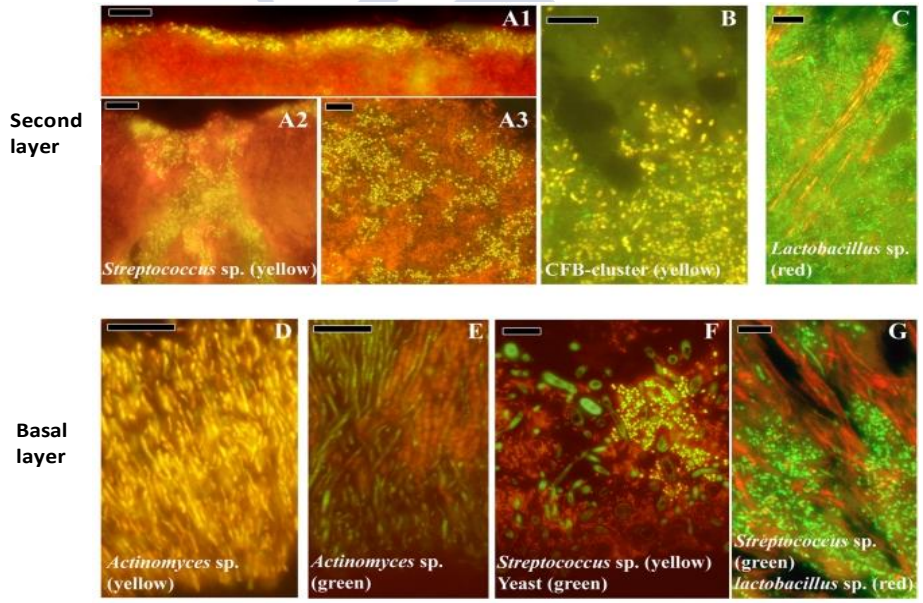


Figure I.3. Localization of the most abundant species in supragingival biofilm. *Streptococcus* spp. (yellow) form a thin band on top of the biofilm (A1), almost engulfed in the biofilm (A2) or present as small cells scattered through the top layer (A3). (B) Cells from the CFB-cluster of bacteria in the top layer of the biofilm, without defined structure. (C) *Lactobacillus* spp. (red) forming long strings through the top layer. (D) *Actinomyces* spp. (yellow) plaque attached to the tooth. (E) *Actinomyces* spp. (green) and cocci forming initial plaque. (F) Multispecies initial plaque composed of *Streptococcus* spp. (yellow), yeast cells (green) and unidentified bacteria (red). (G) *Streptococcus* spp. (green) and *Lactobacillus* spp. (red) forming initial plaque. Black holes might be channels running through the biofilm (Zijnge V et al., 2010).

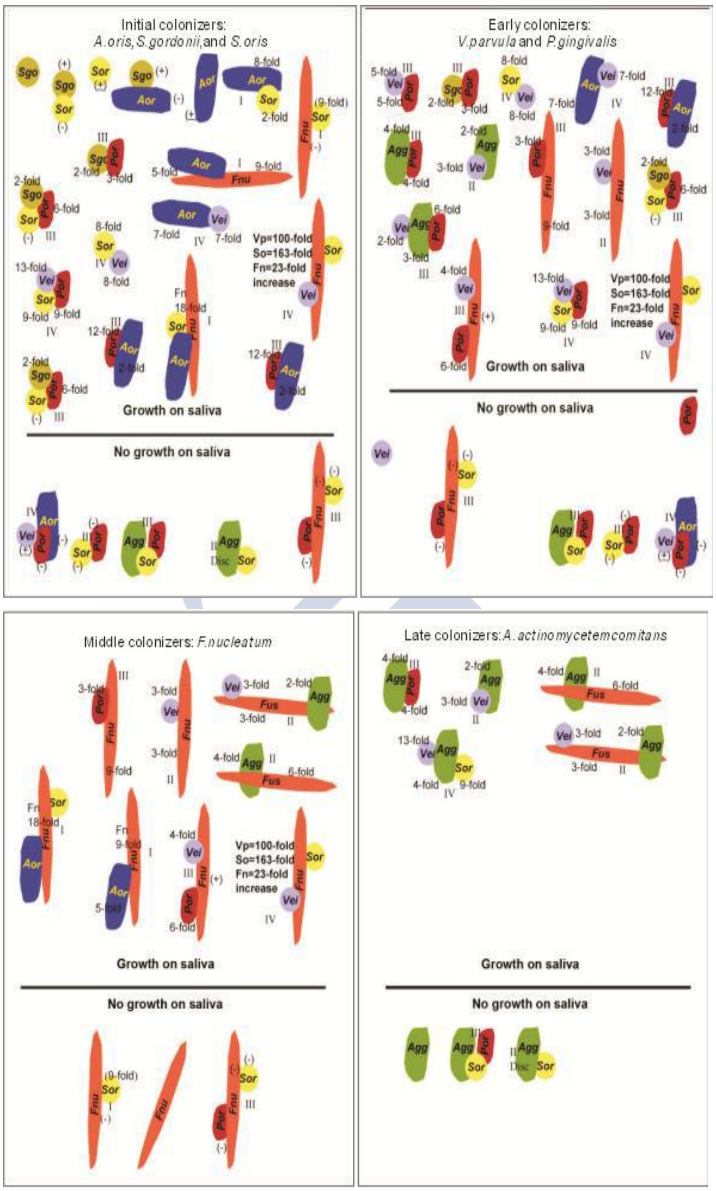


Figure I.4 Salivary growth of oral biofilm colonizers. The pictures show bacterial colonizers that grow and do not grow in saliva as sole nutrient source when incubated individually, as 2- or as 3-species communities (Kolenbrander PE, 2011). Individual bacterial species are depicted with different colour and shape and include Aor: *A. oris*; Agg: *A. actinomycetemcomitans*; Fus: *F. nucleatum* (Fn); Por: *P. gingivalis*; Sgo: *S. gordonii*; Sor: *S. oralis* (So); Vei: *V. parvula* (Vp). The amount of growth of each species between 4 and 18 hours of incubation in a flow cell is given as "fold" increases. Species that grew less than 2-fold are indicated as (+); species that did not grow in pairings or three-species communities are indicated as (-).

The following differences have been described between healthy and cariogenic oral biofilms: (1) the type of microorganisms present in the ecosystem, favoring the imbalance towards disease. In a study of *in vivo* dental biofilm in initial and mature plaque for caries-free and caries-bearing individuals, Thomas RZ et al. (2012) found species like *Rothia dentocariosa* and *Scardovia inopinata,* which were absent from all caries-free sites, but appeared in 50% of the caries-active sites; (2) The metabolic profile of the dental ecosystem, such as diet-dependent nutrient fermentation and environmental acidification, responsible for instrumental changes that occur in the microbiota during caries initiation (Takahashi N and Nyvad B, 2011); (3) The inter-species co-aggregation pattern happening not only in the biofilm but also in saliva (Figures I.2 and I.4) which may influence the attachment and growth process; (4) the microbiome-host interaction in saliva, as this will condition bacterial colonization on the tooth surface and the subsequent development of biofilm (Zaura E et al., 2014).

I.3.2. Pathogens of Dental Caries

Different microorganisms have been associated with dental caries through history, describing their possible implication in the disease from the perspective of a unimicrobial to a polymicrobial etiology. For several decades, it has been established that the principal infectious agent involved in the onset of dental caries is *Streptococcus mutans* (Loesche WJ et al., 1975; Loesche WJ, 1980). The following virulence or pathogenicity features of *S. mutans* have been described (Cawson RA, 2009): (1) Acidogenecity, *S. mutans* can ferment dietary sugars and mainly produce lactic acid as final product of the metabolism, causing a decrease in pH and resulting in enamel demineralization. (2) Acidurity, understood as the capacity of *S. mutans* resist the acidity of an environment, a property that is very necessary to survive and develop at low pH, displacing potential competitors. (3) *S. mutans* produces dextranases and fructanases

enzymes that are capable of metabolizing extracellular polysaccharides, which contribute to the acid production and constitute a substratum in the periods with less oxygen supply (Hamada S and Slade HD, 1980). (4) *S. mutans* has a short post-pH effect that is it needs a short time to recover its activity of habitual growth after being subjected to a low pH. (5) Adhesion capacity, due to the presence of surface adhesins and to the production of insoluble glucans that contribute to biofilm complexity and impede salivary protection (Hamada and Slade, 1980)

Another cariogenic bacteria described widely is *Lactobacillus* spp., which has been related to the progression of carious lesions (principally, in injuries in dentine) (Nyvad B and Kilian M, 1990). *Lactobacillus* spp. are not be able to quickly attach to hard surfaces, so these bacteria are detected in retentive zones, as pits and fissures or deep cavities. *Lactobacillus* spp. live in niches with low pH (Beighton and Brailsford, 1998), and another feature is the tolerance to acid environments, since these bacteria contain the agmatine pathway (Arena ME and Maca de la Nandra MC, 2001), which helps neutralize their cytoplasm pH.

Other studies have demonstrated that *Veillonella* spp. are predominant at all stages of caries progression (Aas JA et al., 2008: Belda-Ferre P et al., 2012) and under high-glucose conditions, and appear to be implied in acid production (Bradshaw DJ and Marsh PD, 1998). Interestingly, consortia between *Veillonella alcalescens* and *S. mutans* were shown to produce more acid than any one of these species separately (Noorda WD et al., 1988), suggesting that synergistic effects probably take place, as it has been demonstrated in other complex microbial communities. Some authors observed carious lesions with absence of *S. mutans*, suggesting that this bacterium could not be a necessary actor for development or appearance of the oral disease; in some of these cases, *Atopobium* spp. were associated with these lesions (Aas JA et al., 2008).

An even more complex scenario was drawn when Tanner AC et al. (2011) proposed *Scardovia wiggsiae* as a clear initiator agent of early childhood caries, and *Bifidobacterium* spp. were also described as important microorganisms of tooth decay in root caries lesions (Mantzourani M et al., 2009). *Candida* spp. have also been involved in the carious process, since they may be present in acidogenic environments. All these findings reflect the polymicrobial character of dental caries, and the convenience of applying advanced microbiological and molecular techniques to improve our understanding of the complex ecosystem implicated in dental caries.

I.3.3. Etiology of Dental Caries: Hypotheses

There are three proposals to explain dental caries based on the role of the dental biofilm in initiation and progression of the disease.

The **Specific Plaque Hypothesis** (Loesche WJ, 1992) proposes that, independently of the diversity of microorganisms comprising the resident plaque microbiota, only a single or very small number of species were actively involved in disease. This proposal allowed to focus efforts on controlling disease by targeting preventive measures and treatment against a limited number of organisms, such as by vaccination or gene therapy, or by antimicrobial treatment. In contrast, the **Non-Specific Plaque Hypothesis** (Theilande E, 1986) considers that disease is the outcome of the overall activity of the total plaque microorganisms. Under this hypothesis, not just those that make acid, but also species that produce alkaline compounds or consume lactate need to be considered. Thus, a heterogeneous mixture of microorganisms could play a role in disease development. In some respects, the arguments about the relative merits of these hypotheses may be partly semantic, since biofilm-mediated diseases are essentially mixed culture (polymicrobial) infections, but the specific

plaque hypothesis would contend that only certain (perhaps a limited number of) species are able to predominate.

The **Ecological Plaque Hypothesis** (Marsh PD, 1994; Marsh PD, 2003) argues that the microorganisms associated with disease may also be present at healthy sites (Figure I.5), but at levels too low to be clinically relevant. Disease would then be the result of a shift in the balance of the resident microbiota driven by a change in local environmental conditions. In the case of dental caries, repeated conditions of low pH in plaque following frequent sugar intake (or decreased sugar clearance following low salivary secretion) would favor the growth of acidogenic and aciduric species, and thereby the appearance of carious lesions.

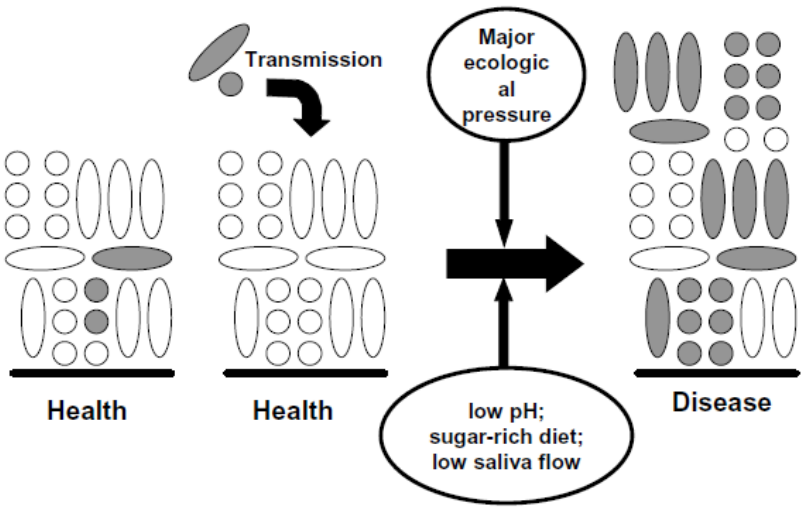


Figure I.5. Schematic representation of the relationship between the microbial composition of dental plaque in health and disease. Potential pathogens (grey) may be present in low numbers in plaque, or transmitted in low number to plaque; both situations may be compatible with health. A major ecological pressure would be necessary for such pathogens to compete with other members of the resident microflora (white), achieving the levels (numerical dominance) required for disease development (Marsh PD, 2003).

I.4. IMMUNOLOGY OF DENTAL CARIES

Oral immunity is the balance system that the human body has to control the microorganisms present in different oral tissues, and fluctuations due to external aggressions. The mouth is a path of entry and exchange with the environment, and is therefore subject to constant fluctuations that must be evaluated and properly controlled by the immune system (Boman HG, 2000; Wu RQ et al., 2014; Zasloff M, 2002). This would prevent the passage of harmful substances and microorganisms recognized by our defense mechanisms as potentially dangerous, avoiding the disease development (Marcotte H and Lavoie MC, 1997).

I.4.1. Immune Role of Saliva

Human saliva is part of the mechanisms of natural or innate immunity of the oral cavity. Its viscosity makes it difficult for the adhesion of microorganisms. In addition, salivary flow exerts a cleaning function, provides antibacterial proteins, enables high buffering capacity (remineralization) and helps neutralize acids (Prabhakar A et al., 2009).

The composition of saliva is water (94%) and solid content (6%). This is 1% suspension, consisting of desquamated epithelial cells, white blood cells, bacteria, yeasts, fungi and viruses; the rest of the solid content is dissolved and formed by organic metabolites (immunoglobulins, proteins, glucose, urea, citrate, enzymes, thiocyanate and lactate) and inorganic, including Ca, Na, P, Cl, K, CO_3H^-, F, O_2, CO_2 y N_2 (Dodds MW et al., 2005). Total salivary proteins constitute 0.3% of the volume, are elements necessary for interaction with microorganisms and essential in the development of oral innate immunity.

I.4.1.1. Salivary Compounds

The components of the immune system are related to the emergence and development of dental caries, due to its interaction with the microbial ecosystem established in the oral cavity (Amerongen AV and Veerman EC, 2002; Feller L et al., 2013). They are produced by the mucosal cells and salivary glands and released to form part of the salivary components such as: human defensins (alpha-defensin 1-3 and beta-defensins 1-3), antimicrobial proteins (LL-37, catelicidina, calprotectin, lactoferrin) and immunoglobulins (IgA, IgG and IgM) (Figure I.6). All of these metabolites are necessary to control the microbiota and its fluctuations in different clinical situations. Their concentrations can vary depending on circadian rhythm of the individual and age, as well as the changes that occur in the tissues that produce them (Costalonga M and Herzberg MC, 2014).

Other salivary compound related to tooth decay is urea. It is an organic substance derived from protein metabolism and that generate the release of ammonium, which induces toxicity on the bacteria and can regulate pH (Hay DI, 1995; Reyes E et al., 2014). Salivary calcium and phosphorus, whose concentrations have been correlated with the risk of tooth decay.

There is no clear scientific evidence on the anticaries role played by specific IgA (Fidalgo TK et al., 2014). Some authors have demonstrated that the levels of specific IgA showed a modest inverse relationship with caries risk, while other studies reported an inverse relationship between specific IgA antibodies in saliva (for example, anti-*S. mutans* IgA) and caries status (Malcolm J et al., 2014). However, in two series, the authors found that caries was related to increased levels of specific IgA and even there are five studies, in which no

relationship between specific IgA and caries was detected (Leone CW and Oppenheim FG, 2001; Kirstilä V et al., 1998). Although IgG and IgM may be compensatory in IgA deficient subjects, there is insufficient evidence to establish a role for these immunoglobulins in caries risk (Koga-Ito CY et al., 2003).

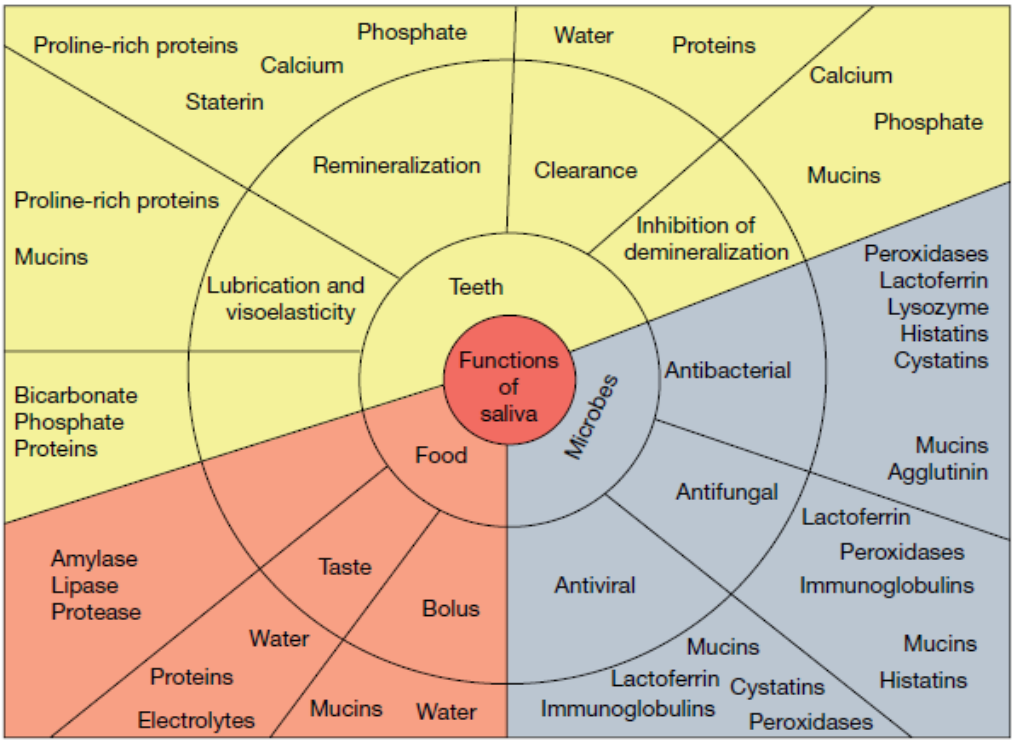


Figure I.6. Multiple functions of saliva in relation to teeth, food intake and oral microbiology. As shown, both the quantity of saliva and its inorganic and organic composition have a wide array of functions which may influence oral health (figure from Van Nieuw Amerongen A et al., 2004)

The neutralization of oral microorganisms by the immune system has been seen in subcutaneous immunization studies with *S. mutans*, where the organism was found to be phagocytosed and undergoes antigenic processing by macrophages. Polymorphonuclear leucocytes have specific receptors for the Fc part of IgG to enable the antibody bound to *S. mutans* to adhere the

polymorphonuclear membrane. The complex is then internalized in vacuoles called phagosomes, which may combine with the lysosomes of the leucocyte to form phagolysosomes. The microorganism will then be killed by the action of lysosomal enzymes (Kaur A et al., 2013).

I.4.2. Immune Role of Dental Tissues

In terms of the tissues where decay occurs, enamel has no immunological capacity to test and respond to the degradation and colonization of microorganisms, due to its inert nature. Thus, any immune response to prevent enamel degradation must take place before reaching the tissue, through the potential protection of saliva, which I have studied in Chapter 4 of this Thesis. The amelodentin barrier is in the dentinopulpar complex, and it is only there when the tooth is first capable of recognizing the bacteria and activates innate immunity, and subsequently the acquired immunity to protect the pulp and thus safeguard dental vitality (Goldberg M et al., 2011; Bleicher F, 2014).

During caries infection, oral bacteria degrade enamel and dentin and trigger an innate immune response in the dental pulp through the diffusion of bacterial by-products into dentin tubules. This response may eliminate the insult and block the route of infection when accompanied by dentin neoformation within tubules and/or at the pulp–dentin interface. Pathogen invasion may result in excessive and deleterious pulp immune response, irreversible acute inflammation, tissue necrosis, and microbe dissemination through blood vessels. Previous data have revealed that bioactive molecules, mostly of the Transforming Growth Factor-beta/Bone Morphogenetic Protein family, induce

dentin formation at the pulp-dentin interface. However, this formation is greatly impaired by the increase of pulp inflammation (Goldberg M et al., 2011; Bleicher F, 2014).

I.4.3. Microbial-Host Interaction to Treat Dental Caries

From an applied point of view, various immunological components have been considered as possible targets in microbial control of tooth decay, although they have mainly been developed against *S. mutans* and *S. sobrinus* (Table I.1).

Table I.1. Function of salivary immunoglobulins in dental caries (Matsumoto-Nakano M et al., 2007).

Isotype	Steps in caries pathogenesis	Mode of action	Antibody specificity
sIgA	Adherence to salivary pellicle	Blocking of adhesion receptor interaction	AgI/II
		Reduction of hydrophobicity	Surface antigens
		Agglutination and clearance	Surface antigens
	Binding to early colonizers	Blocking of adhesion receptor interaction	AgI/II
	Sucrose dependent accumula	Inhibition of glucan production	GTF
		Inhibition of substrate binding	Catalytic region
		Inhibition of polymer synthesis	Glucan Binding region
		Blocking of adhesion	GTF
			GBP
	Acid production and other me	Blocking of glucose uptake	Not Known
		Synergism with	
		Peroxidase (inhibition of acid production)	Not Known
		Lactoferrin (inhibition of iron acquisition)	Iron uptake molecules
IgG	Colonization of cervical tooth	Opsonisation and phagocytosis	AgI/II other surface antigens?
	Invasion dentinal tubules	Inhibition od collagen binding	AgI/II

Several stages in the molecular pathogenesis of dental caries are susceptible to immune intervention. Antibodies could block the receptors necessary for colonization (e.g., adhesins) or accumulation (e.g., glucan binding domains of GBPs and GTF), or inactivate GTF enzymes responsible for glucan formation (Matsumoto-Nakano M et al., 2007).

Adhesins from two principal human pathogens are antigen I/II, PAc, or P1 (for *S. mutans*) and SpaA or PAg (for *S. sobrinus*). Antigen I/II (AgI/II) has been found both in the culture supernatant as well as on the *S. mutans* cell surface. Antibodies directed to the intact AgI/II molecule or to its salivary-binding domain block adherence of *S. mutans* to saliva-coated hydroxyapatite (Matsumoto-Nakano M, 2007).

The ability of *S. mutans* to bind to glucans is mediated by cell-wall-associated glucan-binding proteins (GBP). Many proteins with glucan binding properties have been indentified in *S. mutans* and *S. sobrinus* (Mitchell TJ, 2003). Each glucan-binding protein has the ability to bind α 1-6 glucan (Matsumoto-Nakano M, 2007). *S. mutans* secretes at least three distinct proteins with glucan-binding activity: GbpA, GbpB and GbpC.

I.5. METHODOLOGIES TO STUDY ORAL MICROBIOLOGY

For over a century, the cornerstone for studying microorganisms has been pure culture. However, a large portion of human-associated microorganisms have not been cultured to date. In the oral cavity, these include whole phyla like TM7 or OP1, which appear to be very frequent in saliva and plaque samples, and for which not a single member has been isolated in pure culture yet. In general, it has been estimated that about half of oral bacteria correspond to yet uncultured species (Marsh PD et al., 2011; Munson MA et al., 2002). Although the last years have seen important efforts trying to develop new culture media to improve the diversity of species recovered by culturing (Vartoukian SR et al., 2010; Nichols D et al., 2010), this gives still a very limited

picture of microbial diversity. With the arrival of DNA-based molecular methods, whole new array of bacterial groups were identified in human oral samples Paster BJ et al., 2001, Aas JA et at., 2008). In a comparative study where different environmental and human samples, including the oral cavity, were analyzed by traditional culture and Sanger sequencing of cloned PCR-amplified 16S sequences, many species were uniquely found by PCR and passed undetected by culture (Donachie SP et al., 2007). Surprisingly, the opposite pattern was also found, which led the authors to claim that some species could be missed by PCR and be detected by culture, underlining the value of traditional methods. Although this interesting work highlights the known amplification bias of PCR (Gonzalez JM et al., 2013), the main reason for not detecting some bacterial species by PCR was the low number of 16S sequences that were sequenced by the original cloning method, which rarely passed one hundred clones. In fact, with the arrival of high-throughput sequencing like 454 pyrosequencing or Illumina sequencing, the cloning step could be obviated, and thousands of sequences could be obtained per sample (Keijser BJ et al., 2008). When traditional culture (using different culture media and growth conditions) and pyrosequencing was applied to the same human samples, the diversity found by sequencing was dramatically higher than that obtained by culture (Benítez-Páez A et al., 2013), strongly indicating that the "omics" era has provided a unique opportunity to unravel the total diversity found in oral samples (Figure I.7), enabling the study of oral diseases etiology.

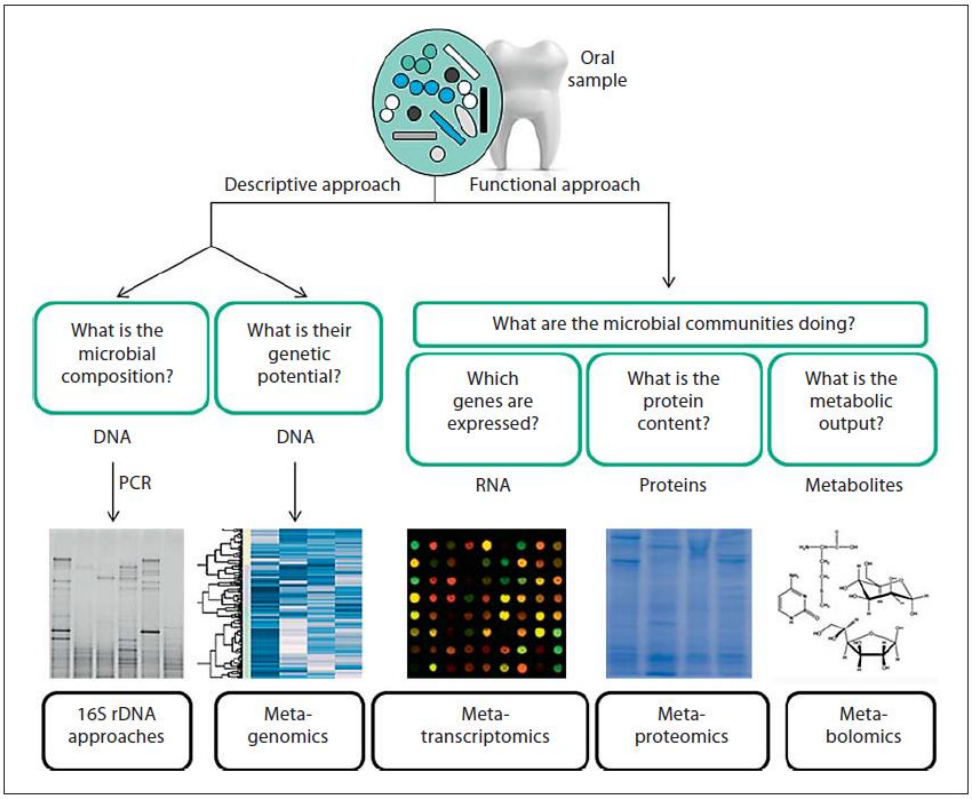


Figure I.7. Schematic representation of different 'omics' approaches to study oral microbial communities. DNA can be extracted from an oral sample to determine taxonomic composition by different 16S rDNA approaches or to study the functions coded by the total gene content of the community (i.e. the metagenome). If RNA is extracted and sequenced, the obtained sequences (i.e. the metatranscriptome) will represent the global pattern of expressed genes in the active portion of the bacterial populations. The total protein and metabolic output of the community will be obtained by analyzing the metaproteome and metabolome by different mass spectrometry techniques. If the metagenomic approach provides information about the total genetic repertoire of the microbes, the metatranscriptomic, metaproteomic and metabolomic approaches give insights about the active organisms and functions at the moment of sampling. The 16S rDNA-based methodologies are cheaper than 'omics' approaches but do not provide information about the functional capacity and output of the oral community (Nyvad B et al., 2013).

I.5.1. OMICS and its Applications

Recent advances in Next-Generation Sequencing (NGS) have been crucial to know deeply the microorganisms present in a specific environment and to understand changes between healthy and disease conditions in a complex ecosystem. In this Thesis, I have applied 16S rDNA pyrosequencing, metagenomics and metatranscriptomics approaches (Figure I.7) to compare the healthy and dental caries microbiome and its interaction with the host.

Depending on study requirements, a project may be performed by different technologies. The length and coverage (quantity of sequences) represent important differences between technologies. Pyrosequencing by Roche (Margulies M et al., 2005) and Illumina by Solexa (Bennett S, 2004) are the principal "2nd-generation" platforms used nowadays for sequencing amplicons or whole DNA. Other, less successful 2nd-generation technologies include SOLiD (McKernan KJ et al., 2009) or IonTorrent (Rothberg JM et al., 2011). All these techniques are based on an initial library preparation step which implies a PCR amplification of the sample, with the associated biases this may introduce to the final results (Schwientek P et al., 2011). Recently, single molecule "Third Generation Sequencing" platforms are being developed, which aim at enhancing previous features in relation to length and high coverage of sequences reads without the amplification biases from second generation machines. These include Pacific Biosciences (Eid J et al., 2009) and Oxford Nanopore technologies (Clarke J et al., 2009), which by the end of this Thesis were already showing promising improvements in error rates, which in conjunction with their long read lengths of several Kbp, will most likely make them the methodologies of the future (Table I.2).

Table I.2. Characteristics of first, second and third generation sequencing technologies (Schadt EE et al., 2010).

	First generation	Second generation[a]	Third generation[a]
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

[a]There are many TGS technologies in development but few have been reduced to practice. While there is significant potential of TGS to radically improve current throughput and read-length characteristics (among others), the ultimate practical limits of these technologies remain to be explored. Furthermore, there is active development of SGS technologies that will also improve read-length and throughput characteristics.

Within second-generation sequencing methodologies, the longer length of pyrosequencing reads, especially with the recently launched titanium-plus system (over 650 bp on average) is normally sufficient for a direct recognition of gene function by sequence similarity, allowing a comparison of the functions encoded by the oral microbiota from different individuals (Belda-Ferre P et al., 2012).

I.5.1.1. NGS: Pyrosequencing by 454 Technologies

The pyrosequencing approach to study microbial diversity can be divided in two forms depending on the type of input DNA. In the direct metagenomics approach, when DNA is not amplified, a preliminary step of processing and adaptor ligation to generate a "Shotgun" library consisting of small genome fragments is required (Figure I.8). This mode requires the application of a

physical process, in which the DNA breaks up into fragments of between 500-1000 base pairs. Two adapters A and B are then added to each end of the fragments obtained by nebulization. This step is performed using ligation (Margulies M et al., 2005).

Another variant involves the direct sequencing of PCR products that have been amplified with primers containing built-in A and B adapters, which makes unnecessary any step of spraying and ligation. In the approach used in the current Thesis, the adapter A was built-in in the forward primer (allowing unidirectional sequencing from that end) and the B adapter on the reverse primer (allowing attachment to the pyrosequencing microspheres). The procedure is, from this point onwards, equal in both variants (Kelly BT et al., 2007).

The DNA undergoes PCR amplification in an oil emulsion that contains individual nanoparticles (Figure I.8.c), has and each of this beads, after the emulsion PCR (emPCR) ends up reaching thousands of identical single-stranded DNA molecules (Metzke ML, 2010). Nucleotides are added sequentially and in a known order. This addition causes an enzymatic cascade that begins when the polymerase facilitates the incorporation of a nucleotide in a range of free DNA, generating a molecule of pyrophosphate (PPi). The sulfurylase converts this PPi to ATP using adenosine-phospho-sulfate. The ATP is hydrolyzed by the enzyme luciferase, producing oxoluciferine and emitting light, ending the enzymatic cascade that occurs in the process (Ahmadian A et al., 2006).

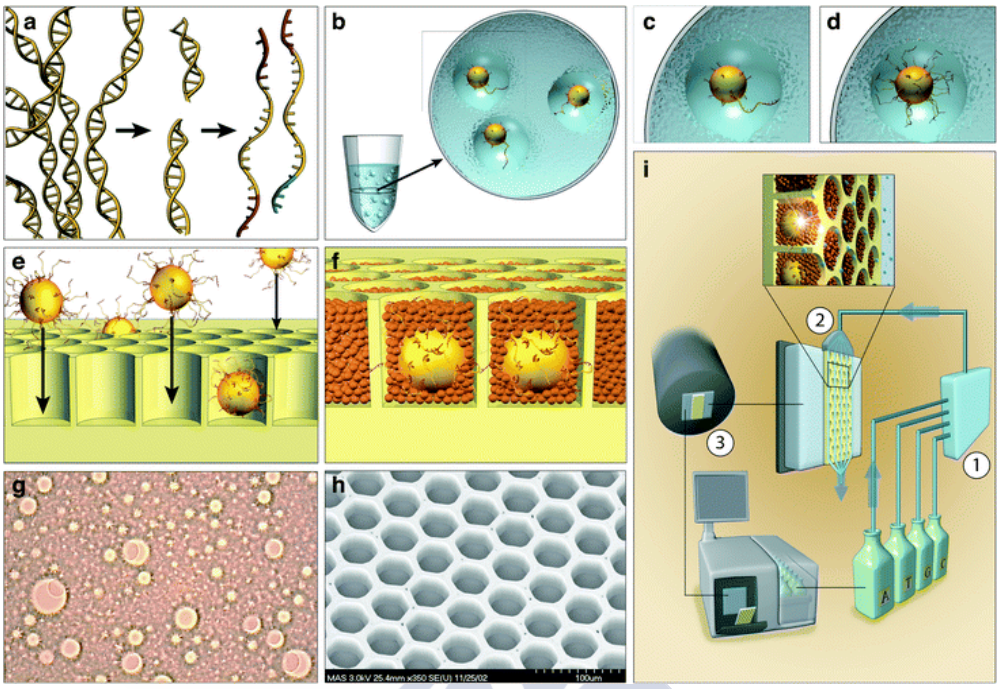


Figure I.8. Scheme for pyrosequencing process. (a) Genomic DNA is fragmented, ligated to adapter DNA sequences and separated into single strands. (b) DNA is immobilized on microbeads coated with a PCR primer such that the majority of beads carry at most a single DNA fragment. (c) The beads are then compartmentalized in a thermostable emulsion along with the ingredients of a PCR reaction and the mixture is thermocycled as in conventional PCR, leading to beads coated with ten million copies of the initial DNA fragment (d). The beads are recovered from the emulsion, the DNA strands denatured, and the beads (now carrying millions of single-stranded copies of the starting DNA fragment) are deposited in the wells of a fibre-optic slide (e). Smaller beads, carrying the enzymes required for pyrosequencing, are deposited into each well (f). Microscope photograph of a thermostable emulsion (g). Scanning electron micrograph of a fibre-optic slide, showing fibre-optic cladding and wells before bead deposition (h). A general scheme of the machine microfluidics is shown in (i). (taken from Kelly BT et al., 2007).

A "PicoTiter" plate containing over a million wells is introduced into the sequencer, which automates the process of sequencing, image capture and interpretation (Figure I.8.g). The sequencer pours automatically over the wells the necessary reagents and nucleotide type cyclically. The DNA polymerase adds one or more nucleotides depending on the sequence that acts as a mold and light will be issued with proportional intensity to the number of nucleotides incorporated into the new chain. The emission of light is detected by a highly

sensitive CCD camera. The process is repeated cyclically with another type of nucleotides and washes between each cycle, which eliminate the excess of nucleotides and reagents, and the process with another type of nucleotide in a cyclic way is repeated until finishing the synthesis of a chain complementary to the mold. The result is the sequencing of fragments that exist in each well.

I.5.1.2. PCR of 16S rDNA to Study Bacterial Diversity

Approaches based on amplification and sequencing of 16S rRNA gene of complex communities such as the oral ecosystem have revolutionized the study of the human microbiome (HMP Consortium 2012) and allowed a complete description of oral bacterial populations (Crielaard W et al., 2008; Zaura E et al., 2012). From tens of sequences per sample obtained in traditional cloning studies, the 16S pyrosequencing approach allows to study thousands of sequences per sample (Cabrera-Rubio R et al., 2013). However, it has been recognized that the PCR step introduces biases due to the over-amplification of some taxonomic groups and the underrepresentation of others, especially under some experimental conditions like high annealing temperature (Sipos R et al., 2007) or the use of different primer pairs (de Lillo A et al., 2006). In the study of dental caries, it has been proposed that different bacterial consortia may be responsible for specific features of the disease. Consequently, the identification of specific microorganisms associated with dental caries could prove to be elusive and the analysis of a high number of sequences from caries samples taken at specific site locations has been recommended (Takahashi N and Nyvad B, 2011).

Bacterial 16S rRNA gene contains regions that are highly conserved among all bacteria –allowing the design of "universal" primers"- and

hypervariable regions which allow discerning between different bacterial types. These two key attributes combined with amplification polymerase chain reaction (PCR) the study of communities of microorganisms in different environments, including the microbial communities of human beings (HMP Consortium, 2012).

Amplification of DNA by PCR is an enzymatic reaction that generates multiple copies of the initial hereditary material through a specific number of temperature cycles. Each cycle doubles the amount of initial DNA, so it is an exponential amplification of genetic material according to the number of cycles used. The PCR requires a set of reagents which, together with the thermostable polymerase enzyme, allow the exponential amplification of DNA from the starting material. This includes using dNTP's, which uses the enzyme polymerase to complete and create the DNA strand mold; a buffer to maintain the optimum pH of the enzyme during the reaction and a divalent ion as $MgCl_2$, used as a cofactor for the polymerase. In addition, two complementary primers to each of the two initial DNA chains are required (Iverson SL and Maier RM, 2009).

In the present Thesis, we have used 16S rRNA universal primers, that is, the forward primer 8F and reverse primer 533R (Chapters 1, 2 and 4), which cover the hypervariable regions V1, V2 and V3 of the 16S gene. The used primers were individually modified with two adapters (adaptor A for forward primer; adaptor B for reverse primer), which are required in the steps of amplification, selection and sequencing related to the pyrosequencing process. In addition, the forward primer 8-27F has a unique sequence of 8pb that acts as a "barcode" to identify the amplicons of each sample after sequencing. That way, different samples can be mixed in the same pyrosequencing well, which allows a considerable cost reduction while still obtaining a high number of reads per sample (Cabrera-Rubio R et al., 2012). At the time of the amplification of

RNA-based samples performed in Chapter 3, improvements in the pyrosequencing technology allowed the sequencing of longer PCR fragments Thus, I have used a different set of reverse primers, located at position 788, to amplify over 750 basepairs, encompassing the hypervariable regions V1 through V5, allowing a more reliable taxonomic assignment. The PCR products are purified to remove primers and other reagents used in the PCR reaction, prior to its pyrosequencing (see, for example, Benítez-Páez A et al., 2012).

I.5.1.3. Metagenomics Approach

"Bona fide" metagenomics approach by direct sequencing of total DNA allows to study not only what microorganisms are in the studied community, but also all of genes present, enabling the description of the functional profile of the ecosystem (Belda-Ferre P et al., 2012; Nyvad B et al., 2013).

Metagenomics has several objectives: to describe the biodiversity of a certain ecosystem (in both terms, qualitatively and quantitatively), to study the most relevant functional activities, and if possible, to obtain an overview of the relationships between the microorganisms. Initially, the used approaches included the cloning of DNA from the complex community in a vector that gets introduced in a culturable laboratory species like *Escherichia coli*, performing either a sequence-based or function-based screening of functions of interest (Gilbert JA and Dupont CL, 2011; Foster JA et al., 2012). This approach was first applied in the oral cavity by cloning plaque DNA in fosmid vectors, whose expression in *E. coli* allowed the identification of multiple antibiotic resistance genes (van Hoek AH et al., 2011). However, the cloning of environmental DNA has been shown to have an important bias by impeding the cloning of genes which are toxic for the host. Thus, metagenomics reaches its full potential when combined with the use of new techniques of mass sequencing, where the sequencing of metagenomic DNA can be performed directly, eliminating the PCR

and cloning biases (Ghai R et al., 2010), an approach which has been used in Chapter 2 of this Thesis.

PCR of 16S rDNA and direct metagenomics approaches are based on DNA studies, which analyze whole bacterial community without considering whether the microorganisms are active or quiescent, alive or dead. Therefore, these techniques allow the description of the total microbial composition or genetic potential in the environment (Figure I.8), regardless of the microorganisms or gene functions that are actively contributing to the community (Nyvad B et al., 2013).

I.5.1.4. Metatranscriptomics Approach

The RNA analysis allows identifying active members of a microbial community and their genes expressed under given circumstances. This approach is known as metatranscriptomics. The pioneering RNA-based studies were performed with environmental samples where total extracted RNA was reverse-transcribed to cDNA, which was then sequenced by one of the NGS technologies (Frías-López J et al., 2008). A limitation of this approach comes from the short RNA half-life and the high percentage of rRNA present in bacterial samples, which typically accounts for over 90% of total RNA, and different methods have been applied to enrich the sample in mRNA before sequencing (Moran MA et al., 2013). When analyzing RNA instead of DNA, actively transcribed genes can be revealed, obviating those bacteria that are present in the sample but not actively transcribing genes under given conditions (Gentile et al., 2006). Metatranscriptomic approaches have recently began to be applied in faecal human samples (Turnbaugh et al., 2010, Gosalbes MJ et al., 2011), *in vitro* oral models (Frias-López J and Durán-Pinedo A, 2012) and dental plaque

samples from subgingival and supragingival sites (Durán-Pinedo A et al., 2014; Benítez-Páez A et al,. 2014), but not from caries lesions.

Thus, a combination of different metagenomic and metatranscriptomic approaches may be necessary to study the taxonomic composition, the functional output and the actively expressed genes in a given biological condition.

Along this Thesis, I have used pyrosequencing approach based on Roche technology for 16S rDNA gene studies to identify bacteria present in different oral sites and those associated with health and dental caries (Chapters 1 and 2), as well as in the study of bacterial-host interactions mediated by immune recognition in caries-free and caries-active individuals (Chapter 4). I have used a metagenomics approach to determine the functional profile through the direct sequencing of total DNA with the purpose of classifying healthy and caries-bearing patients (Chapter 2).

Also, I have analyzed the RNA fraction of total bacterial populations at different stages of dental caries progression in order to identify functionally active bacteria associated with disease development (Chapter 3). The different culture-independent approaches applied have aimed to contribute to our knowledge about the microbiology of dental caries, its etiology and interaction with the immune system.

I.5.1.5. Bioinformatics Approach to Analyze NGS Oral Samples

Bioinformatics is a vital component of microbiome studies. As the cost of sequencing continues to decrease every year, the main bottleneck for studying complex microbial communities becomes the analysis of a large amount of sequence data in the context of databases continuously increasing in size. Firstly, sequences obtained from the sequencer must be filtered by size and quality to carry out a correct and efficient taxonomic assignment. This is mainly due to two reasons: first, the values of quality tend to be lower as it reaches the end of sequences, because the intensity of the signal falls gradually (Claesson MJ et al., 2010). In addition, the 454 methodology provokes assignment errors in homopolymeric regions, which are generated since the intensity of the light is not exactly linear when several nucleotides are joined in a single flow, and therefore the signal tends to become saturated. Thus, for the machine software it is difficult to distinguish over 8 consecutive nucleotides of the same type. For this type of diversity studies, the best option is to filter out low-quality sequence ends, not only on average, but mainly on sliding windows at the end of the reads, by a process known of as "end trimming", which can be performed by tools like Galaxy (Blankenberg D et al., 2011). In addition, a length criterion should be used to establish the minimum number of base pairs of the obtained sequences so that the results are statistically correct. In this sense, it has been estimated that a big improvement in taxonomic assignment is reached with sequences longer than 250 bp (Claesson MJ et al., 2010), which is the minimum length used in the analyses of this Thesis. When the 454 technology has allowed longer PCR products to be sequenced (Chapter 3), a filtering length of 400 bp has been used. A detailed description of the quality filtering steps and potential biases in the analysis of 16S sequences has been provided by Schloss PD et al. (2011).

It is also crucial that in each of the separate samples chimeric sequences should be removed, which are artifacts composed of at least two sequences from different parental sequences. During the PCR amplification, the chimeric amplicons may arise due to amplifications that have not completed the expansion stage. These amplicons are shorter than those expected and they can act as primers in the next step, being reamplified in subsequent steps, causing a distortion of results. If they are not removed, chimeras may inflate estimates of diversity, and several programs are available to perform this filtering step, like the software Ballerophon (Huber T et al., 2004). Finally, after taking account of the previous considerations, a taxonomic analysis of all samples can be performed against one of the databases of 16S sequences, such as Greengenes, Ribosomal Database Project (RDP) or Silva. In this Thesis, I have performed taxonomic assignment against the RDP database, using the Bayesian RDP classifier to assign each read to a phylum, class, family and genus (Wang Q et al., 2007). At the species level, results can be analyzed using tools that take into account alignments between sequences, for example BLAST, using stringent criteria like long alignments and high sequence similarity, to minimize the frequency of false positives (Camelo-Castillo A et al., 2015).

In addition to the taxonomic analysis, there are numerous indexes that estimate the richness and diversity of a bacterial ecosystem (Wang Q et al., 2007). For this analysis, the sequences of 16S rRNA with a threshold of identity >97% are considered operational taxonomic units (OTU), which represent a consensus threshold for 16S gene sequences belonging the same species (Sogin ML et al., 2006; Yarza P et al., 2008). In the present Thesis, the ChaO (richness) and Shannon (diversity) indices were selected as measures of bacterial diversity in each sample. The Chao index represents the estimated number of OTUs at a given level of identity, whereas the Shannon index indicate

the level of evenness between the species present (high values) or dominance of a few species in the community (low values).

The Shannon index is a statistical index that assumes that all species are represented in a sample in a randomized fashion. In the Shannon index, p is the proportion (n/N) of individuals of a species in particular found (n) divided by the total number of individuals who are (N). When richness is concentrated to a species, and the remaining members are very rare (although there are many of them), the Shannon index is approaching zero (Hill TC et al., 2003).

The bacterial composition can be studied and displayed through the analysis of all samples by Principal Coordinates Analysis (PCoA). The PCoA shows how data contribute to explaining variance by providing a grouping of the samples based on the bacterial composition at a 97% sequence identity. For comparing the diversity, the PCoA is estimated with a phylogenetic approach, which takes into account both taxonomically assigned and unassigned readings. Regarding the PCoA interpretation, a sample occupies a specific position in a multifactorial space according to its bacterial composition, in such a way that samples with similar composition appear closer together (Lozupone et al., 2006). Statistical comparisons in bacterial composition between samples can be performed by significance tests based on the phylogenetic UniFrac distances (Hamady M et al., 2010). The test provides P-values for multiple comparisons that need to be corrected by multiplying the raw P-value by the number of permutations (Roesch SC et al., 2009).

A useful tool to study and visualize differences in bacterial and gene composition is provided by the heat maps. They consist of a display that simultaneously includes row and column of the hierarchical cluster structure in a data matrix (Wilkinson L and Friendly M, 2008). It includes a rectangular tiling with each tile shaded on a color scale to represent the value of the corresponding element of the data matrix. The rows (columns) of the tiling are ordered such that similar rows (columns) are near each other. On the vertical and horizontal margins of the tiling there are hierarchical cluster trees. It can be used for representing bacterial taxonomy related to healthy and disease or for comparing functional activity of different bacterial communities, as I have used in Chapter 2 of this Thesis. The overrepresentation of functions in a sample *vs* another can be estimated by the false discovery rate method applied to metagenomic sequences, as proposed by the algorithm of White JR et al. (2009), which tests the amount of false-positive predictions (q-values) for a given p-value of significance.

I.5.2. Flow Cytometry

Flow cytometry is a multiparameter analytical technique used for measuring multiple fluorescence emission and dispersion of light ("light scatter") derived from cells or microscopic particles. These are sequentially aligned using a liquid stream laminating, when they are presented one by one and at high speed (thousands of cells per second) against a laser beam to an appropriate length of wavelength (Juan G et al., 1994).

The flow cytometer consists of: a hydraulic system of microfluidics, to introduce and to restrict the cells subjected to analysis by establishing a laminating regime; an optical system, which combines a source of excitation (laser, lenses and prisms to direct the light beam) and a collection system to generate and collect the light signals through optical mirrors and filters that select certain wavelengths and redirect them towards optical detectors. Finally, there is an electronic system responsible for processing optical signals into electrical signals useful for computational analysis (Figure I.9).

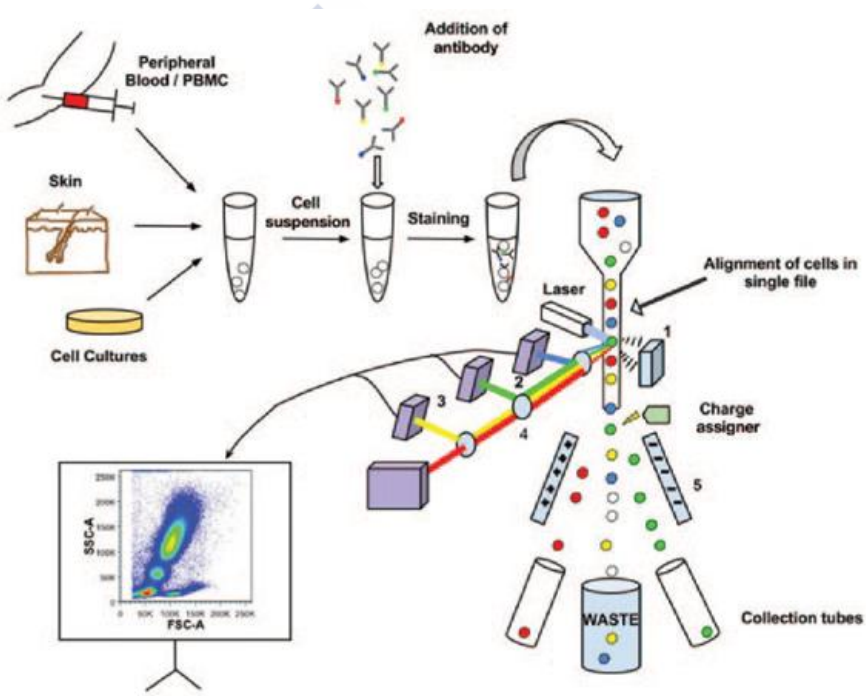


Figure I.9. Schematic representation of a flow cytometer. (1) Forward-scatter detector, (2) side-scatter detector, (3) fluorescence detector, (4) filters and mirrors, and (5) charged deflection plates. Samples are labeled and bacterial cells counted and sorted according to their fluorescence (Jahan-Tigh RR et al., 2012)

The main objectives of this technique consist of identifying and counting defined cell subpopulations in order to achieve their physical separation by electrostatic deflection or "electronic cell sorting" and to measure the functional

ability or any other feature of these cell populations. Therefore cytometry detects cellular-light interaction, as cell deflect incident light and emit fluorescence.

Fluorimetric analysis is based on the process of emitting energy in a chemical molecule or fluorochrome, which absorbs a photon of energy (light) at a certain wavelength and produces an electronic excitation and an electronic transition to a higher level. The return of electrons to their ground state (minimum energy state) results in the emission of a new photon of energy, which is detected. This type of analysis requires the marking of the objects of analysis, cells or antibodies, with a fluorochrome (Guasch RM and O'Connor JE, 1998; O'Connor JE et al., 2001).

The separation takes place by fluorescence of subpopulations with a separator (sorter) that incorporates the cytometer, which can distinguish different wavelengths (Figure I.9). On the other hand, cytometer software allows counting the number of events and performs its filtering for quality.

Therefore, flow cytometry allows quantifying the number of present events and correlating them with the fluorophores that mark populations. In addition, the composition of bacterial populations marked with different fluorophores could be addressed by other methodologies, that would be applied simultaneously, such as metagenomics (Palm NW et al., 2014), as I have applied in the current Thesis for the first time to oral samples.

I.5.3. Diagnostics Methods

Some of the most extended commercial diagnostic methods for the prevention of dental caries are microbiological, based on culture and consists of counting Colony Forming Units (CFUs) of *S. mutans* and *Lactobacillus* spp., which are present in non-stimulated and stimulated saliva and dental plaque samples from the patients. The first bacterium is detected by growth on agar plate Mitis-Salivarius, which contains bacitracin (Gold OG et al., 1973). This is a specific medium, but in advanced lesions can also grow *Enterococcus* spp. and yeasts, although they look very different from *S. mutans*, and their presence indicate contamination. The *Lactobacillus* spp. is detected by growth on Rogosa agar (Rogosa M et al., 1951). It has been established that a count greater than 10^5 UFC of *S. mutans* and *Lactobacillus* spp. per milliliter of saliva refers to a high risk of dental caries (Krasse B, 1988; Andersson MH et al., 1993).

Other methods are related to the pH and evaluate the buffer capacity of saliva, such as Alban test (a simplification of Snyder test). This test is based on the ability of the bacteria to produce acid when a stimulated saliva sample is inoculated in the middle of Snyder medium (Baca P et al., 2011). This medium, with pH 4.7, contains, among other components, glucose, agar and bromocresol green as a pH indicator. The salivary microorganisms metabolize glucose producing acid, which causes a drop in pH that changes the original green color of the media to yellow. The result of this method is measured based on a shift from the original green to yellow and the depth of the colour change. The reading and scoring are carried out at 24, 48 and 72 hours. A very fast change in time (24 hours) is worse than if this is done slowly. Also, there are commercial kits that allow to read the result in minutes, by using a colour strip that indicates acid or alkaline levels of saliva; the values of pH 5 to 5.8 (orange), 6 to 6.6 (yellow) and 6.8 to 7.8 (green), shows high-moderate acidity and low acidity,

respectively, being the first two conditions considered at risk of dental caries (Lingström P et al., 2000).

In addition, the viscosity of saliva is other important feature to keep in mind (Leone CW and Oppenheim FG, 2001), since a high level of fluidity is considered at low risk of dental caries. For measuring the salivary flow, saliva production can be unstimulated or stimulated by paraffin chewing (Saleh J et al., 2015, Jensen JL et al., 1998). In the first case, the amount of drooling saliva produced is measured in a small number of minutes to calculate the salivary flow. In the second case, by quantification of the production of the salivary glands, a low secretion rate is considered when measured flow is in the range of 0.1-0.25 mL/min (normal values= 0.25-0.35 mL/min). Other alternative represents quantifying whole saliva produced during 5 minutes after stimulation with a chewing gum; patients with lower volumes than 3.5 mL (0.7 mL/min) are defined at high-risk while those with greater volumes than 5 mL (1 mL/min), at low-risk (healthy condition). In both cases the production of greater saliva volumes is related to a reduced risk of caries (Tenovuo J, 1997; Leone CW and Oppenheim FG, 2001).

All clinical approaches currently available for the establishment of the risk of decay are considered insufficient (Ruhl S, 2012; Kunin AA et al., 2013). It has been shown that the method for cultivation of microorganisms associated with dental caries (*S. mutans* and *Lactobacillus* spp.) cannot predict if a person is going to have tooth decay due to the fact that these microorganisms are found in both biological conditions, health and disease (Guo L and Shi W, 2013). Also, carious lesions have been diagnosed in the absence of these microorganisms, even by really sensitive methods like PCR (Aas JA et al., 2008). Therefore, these bacteria are not necessary for the development of dental pathology and thus they are not general "risk" biomarkers of dental caries. This makes absolutely

necessary to unravel the microbial etiology of the disease and to establish the relationship between plaque and salivary potential biomarkers. On the other hand, the acidity and quantity of saliva has been associated with a dental caries-related ecosystem. Although these parameters are not always predictive,very skewed values in these parameters can be used for treating high-risk patients and controlling the development of new lesions, especially when the different predisposing factors are considered globally (Figure I.10).

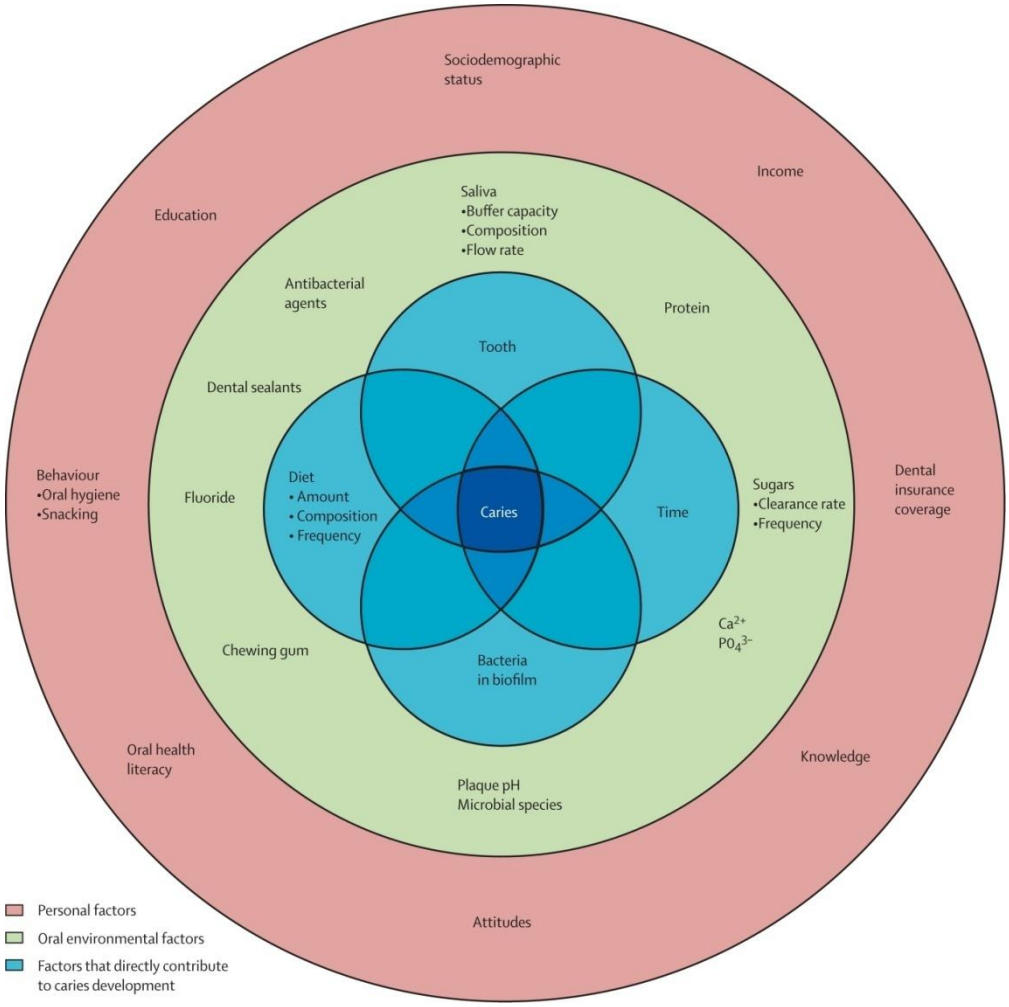


Figure I.10. Schematic illustration of the determinants of the carious process. Those that act at the tooth-surface level are found in the inner (blue) circle. With time, an ecological shift in the composition and metabolic activity of the biofilm (microbial deposit) may result in an imbalance between biofilm fluid and the mineral of the tooth, resulting in formation of a caries lesion (overlap of the two small circles). In the outer (white) ring are listed more distant determinants, which

influence on these processes at individual and population level (Keyes diagram modified from Kidd EA and Fejerskov O, 2008).

Assessing the interaction of all these parameters to the final development of caries has been proposed as a reliable approach to obtain a reliable predictive method (Young DA and Featherstone JD, 2013), although its efficiency has been questioned (Carson SJ, 2013).

I.6. CONCEPTUAL PROBLEMS

Classically, infectious diseases follow Koch's postulates: (1) the pathogen is found in the disease, but absent under health conditions; (2) the pathogen should be related to that disease and not be involved in another; (3) the pathogen must be isolated in a culture from the infected area; (4) the pathogen must produce the disease in an animal by inoculating the microorganism; (5) the pathogen should be isolated from lesions of inoculated animals. Although these initial postulates have been modified after noticing the presence of healthy carriers and in the light of genetics (Fredericks DN and Relman DA, 1996), the general consideration of any microbial-mediated disease as an infectious disease is deeply established in the scientific community. Dental caries has been described throughout history as an infectious disease, in principle related to *S. mutans* (or mutans streptococci) as the principal pathogenic agent (Loesche WJ et al., 1975; Loesche WJ, 1992). Previous culture-based technologies have not been able to clearly show the polymicrobial etiology of dental caries, with numerous species found at various times throughout the disease progression, and depending on the affected tissues and the characteristics of the ecosystem at every stage (Theilade E, 1986; Becker MR et al., 2002; Munson MA et al., 2004). This thesis tries to clarify, thanks to

the application of Second Generation Sequencing Techniques, the different etiological agents involved and the role played by them in the process of tooth decay formation.

The multiple factors that predispose to the development of dental caries are not only of microbiological nature. Apart from the environmental factors such as diet and oral hygiene, the disease-related characteristics of the host must also be considered (Kidd EA and Fejerskov O, 2008). All individuals have inherent factors, both genetic tendency and immunological factors that mark their risk of tooth decay and predispose the presence of a healthy oral microbiome or make it prone to disease. This Thesis aims to cover also the microbial-host interaction in patients with and without tooth decay, in order to assess what tools the host can employ to detect and interact with the cariogenic organisms to ultimately stop the carious lesion, from the start of the lesion in enamel to pulp involvement.

I.7. METHODOLOGICAL PROBLEMS

Sampling for the study of dental caries has been mainly based on saliva (e.g. McInnes P and Cutting M, 2010; Prasanthi B et al., 2014; Bardow A et al., 2014), since it is the oral fluid that envelops all tissues, assumed to be reproducible in different patients, and for its ease and accessibility in the sampling. In recent years, methodological techniques have been developed that allow analyzing in more detail the microbial composition (Wade WG, 2013). In this Thesis, by using molecular methodologies based on high-throughput sequencing, we have studied the composition of different oral samples in relation to the main microorganisms that cause tooth decay. This taxonomic

approach will determine which oral sample is more appropriate to be analyzed in etiological and/or epidemiological studies of dental caries.

Methodologies based on culture have allowed isolating some bacteria that are present in carious lesions, such as *S. mutans* (Clarke JK, 1924) and *Lactobacillus* spp. (Bunting RW, 1937), in chronological order. Subsequently, by the use of cloning techniques, several authors identified in tooth decay other microorganisms that could not get by traditional culture techniques, including (Dewhirst FE et al., 2001) or *Bifidobacterium* spp. (Mantzourani M et al., 2009).

However, these two approaches are insufficient in nature. It has been recognized that traditional bacterial culture is insufficient to cover the full spectrum of microorganisms and their metabolic requirements (Marsh PD et al., 2011; Wade WG, 2002). Although the cloning technique of PCR products represented a step forward in the advancement of knowledge since it avoids cultivation-associated bias (Aas JA et al., 2005), but given that only allows analyzing an average of 50-100 clones, it is not a definitive methodological solution to study dental caries.

For this reason, massive sequencing techniques have opened the way to deepen our knowledge of bacterial populations that are involved in health and disease. In this Thesis, Second Generation Techniques were applied for obtaining thousands of sequences by sample, which allowed us to cover a large portion of the bacterial diversity related to dental caries.

The knowledge of microorganisms present in dental caries is a link that is required to understand the onset and progression of the disease. However,

the taxonomic study of bacterial populations does not reflect what role is played by each member of this complex ecosystem. In this Thesis, by using metagenomics and metatranscriptomics techniques, I studied not only the composition, but also the functionality of each microorganism in the initiation and progression of dental caries.

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Objectives

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Despite the recent advances in oral microbiology, there are still many outstanding questions that need to be addressed in order to understand the pathogenic process of dental caries and propose diagnostic and preventive measures: What is the appropriate oral sample to study dental caries etiology? Is caries disease a tissue-dependent process? Are cavities polymicrobial ecosystems? If so, is the functional output of a microbial community more important than its taxonomic composition in terms of understanding a polymicrobial disease? Does the immune system play a role in caries risk? Answering these questions would allow addressing more applied issues related to caries prevention and treatment. For instance, are immunization and antimicrobial strategies effective in preventing oral diseases? Are we treating polymicrobial oral disorders as infectious single-species diseases? What non-antimicrobial therapeutic strategies are feasible to prevent polymicrobial diseases such as dental caries?

In order to answer these questions, the current PhD thesis includes the following specific main objectives:

1. To study bacterial composition in different niches of the oral cavity, and its relation to the bacterial composition found in saliva samples.
2. To study the microbial composition of cavities and global gene functions present in the oral ecosystem involved in dental caries by a metagenomic approach.
3. To study the active microbiota in different progression stages of tooth decay in order to propose a list of potential etiological agents of dental caries in adults.
4. To study the role of the host immune system in preventing pathogenicity of oral microbiota and determine the specificity of salivary antibodies

against pathogens involved in dental caries.

Secondary objectives of the present thesis include:

1. To determine the appropriate oral sample to study dental caries etiology.
2. To compare bacterial composition and gene functions between initial (enamel) and advanced (dentin) caries lesions.
3. To evaluate the role of *Streptococcus mutans* in initiation and progression of caries disease.
4. To describe the clinical implications of dental caries microbiology.

1

Microbial Geography of the Oral Cavity

Simón-Soro A, Tomás I, Cabrera-Rubio R, Catalan MD, Nyvad B, Mira A. *Microbial geography of the oral cavity*. J Dent Res. 2013 Jul;92(7):616-21.

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1.1 ABSTRACT

We aimed to determine the bacterial diversity of different oral microniches and assess whether saliva and plaque samples are representative of oral microbial composition. We have minutely taken samples from each surface of individual teeth and gingival crevices from two healthy volunteers (112 samples per donor), as well as samples from the tongue dorsum, non-stimulated and stimulated saliva. DNA was extracted from 67 selected samples of each donor and the 16S rRNA gene was amplified by PCR and pyrosequenced to obtain on average over 2,700 reads per sample, which were taxonomically assigned to obtain a geographic map of bacterial diversity at each tooth and sulcus location. The data show considerable differences in bacterial composition between teeth at different intra-oral locations and between surfaces of the same tooth. The most pronounced differences were observed in incisors and canines, where genera like *Streptococcus* were found at 40-70% on the vestibular surface but were almost absent on the lingual sides. Saliva samples, especially non-stimulated saliva, were not representative of supra- and subgingival plaque in the two individuals tested. We suggest that more precise sampling is required to properly determine oral microbial composition and to relate that diversity to epidemiological, clinical and etiological parameters.

1.2. INTRODUCTION

It is well established that the composition of microbial communities varies in different parts of the oral cavity (Segata N et al., 2012). The tongue, teeth, mucosa, palate and gingiva have been shown to harbour a distinctive microbiota by both culture and molecular-based approaches (Aas JA et al., 2005). Thus, oral environments could themselves be heterogenous in their physicochemical properties and therefore host different bacterial repertoires. For instance, Kleinberg I and Jenkins GN (1964) measured salivary flow and pH in different parts of teeth and showed clear contrasts in pH values between teeth and even in surfaces from the same teeth which were in close proximity. This classical work showed that the oral cavity is home to multiple microniches where not only pH, but also oxygen, temperature, or redox potential, among others, can influence the settling of microorganisms and risk of disease (Fejerskov O et al., 1994).

Molecular-based, culture-independent techniques, such as the use of 16S rRNA profiling have provided important new insights into the diversity of the microbiome within the oral cavity (Crielaard W et al., 2011). Massively parallel pyrosequencing is an open-ended molecular approach that allows extensive characterization of microbial populations in a high-throughput, cost-effective manner (Nyvad B et al., 2013).

It has been stated that for gathering complete information on the healthy oral microbiome, microbial samples should be obtained from various ecological niches throughout the oral cavity and from precise and well-characterized disease sites (Nyvad B et al., 2013); however, few studies have been published that apply specific sampling for accurate characterization of the different oral microniches (Haffajee et al, 2009; Zaura E et al., 2009; Segata N et al., 2012; Human Microbiome Project Consortia, 2012).

Given that many oral microbiology studies are based on pooled samples from different teeth, it is imperative to characterize differences in microbial composition among specific oral locations within teeth and gingival crevices, as this could severely influence the interpretation of results. Thus, the aim of the present study was to analyse the bacterial diversity in two healthy individual oral cavities at various intraoral niches by targeted pyrosequencing of the V1-V2-V3 hypervariable regions of the small subunit ribosomal RNA. Sampling locations included different dental surfaces of all teeth and different surfaces of gingival sulcus, across the four quadrants. In addition, the bacterial diversity of non-stimulated saliva *versus* stimulated saliva samples from the same individuals was also evaluated and compared to dental and gingival locations in order to evaluate whether the saliva samples frequently used in etiological and epidemiological studies of oral disorders (Quinque D et al., 2006) are representative of the microbial diversity at the sites where the disease takes place.

1.3. MATERIALS AND METHODS

1.3.1. Donors Selection and Sampling Procedure

Two volunteers were selected for sampling. They were males aged 20-30 years, non-smokers, with 28 teeth excluding third molars, with good dental and periodontal health: in both, absence of caries (non-cavitated level), DMF=0, OHI= 0, GI=1 and CPI=1 (WHO, 1997). They had not been treated with antibiotics in the six months prior to the study nor presented antecedents of routine use of oral antiseptics. The two donors signed a written informed consent and the sampling procedure was approved by the Ethics Committee from the DGSP-CSISP (Valencian Health Authority), with reference 10/11/2009.

In each individual supragingival dental plaque samples were taken 24 hours after toothbrushing from vestibular (buccal) and lingual (palatine) surfaces of 28 teeth, each sample with a different sterile spoon excavator. Teeth were not dried before sampling. From the 56 dental plaque samples collected, 32 were selected for PCR amplification, namely samples from the first incisor, canine, first premolar and first molar from each quadrant. Additionally, fifty-six subgingival samples were taken from the same tooth surfaces, using two sterile absorbent paper points (size 25) per sample, by passing the paper points across each gingival sulcus and avoiding contact with the supragingival dental plaque. Again, thirty-two samples were selected for PCR amplification. Samples were collected and analyzed per lingual and vestibular surfaces in all of teeth. A sample from the tongue dorsum was collected with a sterile spoon excavator across the whole surface with several repetitive strokes to ensure a representative sample. All the samples were taken in the morning, while the donors sat in a quiet atmosphere. A 2 mL non-stimulated saliva sample was also taken 24 h after toothbrushing by drooling saliva into a sterile Falcon 50 mL tube. Two mL of stimulated saliva was collected immediately after, by the use of a sterile paraffin gum.

1.3.2. DNA Extraction, PCR Amplification and Pyrosequencing

DNA was extracted separately from each sample using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies), following the manufacturer instructions, with the addition of a lysozime treatment (Belda-Ferre P et al., 2012). Thus, no physical lysis treatment was performed, which could influence the diversity detected in the extracted DNA (Kuczynski J et al., 2011). A PCR amplification of the 16S rRNA gene was performed with the high-fidelity ABGene DNA polymerase (Thermo Scientific) by the use of universal degenerate primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 533R (5'-GCCTTGCCAGCCCGCTCAGGC-3'), using an annealing temperature of 52 ºC and

20 amplification cycles, to minimize PCR amplification bias. Two PCRs were performed per sample, pooling the two PCR products before purification. In four gingival samples, a PCR product could not be obtained and a nested-PCR was performed, in which the PCR product was purified and used as a template for a secondary PCR in which the primers were shifted 3 bp towards the 3' end and included the pyrosequencing adaptors A and B, following Benítez-Páez A et al. (2013). The 500 bp PCR products were purified with the Nucleofast PCR purification kit (Macherey-Nagel) and further cleaned by AMPure XP beads (Roche) before pyrosequencing. The final DNA per sample was measured by picogreen fluorescence in a Modulus 9200 fluorimeter (Turner Biosystems) so samples could be mixed in equimolar amounts. PCR products were pyrosequenced from the forward primer end only by using a GS-FLX sequencer with Titanium chemistry (Roche). One-eighth of a plate was used for each pool of 20 samples, which were amplified with a different forward primer containing a unique 8-bp "barcode".

1.3.3. Sequence Analysis

Reads with an average quality value lower than 20 and/or with more than 4 ambiguities in homopolymeric regions in the first 360 flows were excluded from the analysis. Read ends were trimmed in 10 bp windows if they had a quality value lower than 20. Only reads longer than 200 bp were considered, as well as those without mismatches in the primer region. Chimeric sequences were detected using Mothur (Schloss PD et al., 2009), and 5.4% of the reads were filtered out as potential chimeras. Singletons were not excluded from the analysis. Sequences were assigned to each sample by the 8-bp barcode and analyzed with the Ribosomal Database Project classifier (Cole JR et al., 2009). Each read was taxonomically assigned down to the genus level using an 80% confidence threshold and reads giving no bacterial hits were excluded from the analysis. All figures and statistical comparisons were performed on samples

from each individual separately. To estimate total diversity, sequences were clustered at 97% nucleotide identity over 90% sequence alignment length and rarefaction curves were obtained using the RDP pyrosequencing pipeline. Principal Coordinates Analysis (PCoA) was performed with FastUnifrac (Lozupone C et al., 2006). The Unifrac analysis compares the 16S-estimated diversity with a phylogenetic approach that takes into account both taxonomically assigned and unassigned reads.

1.4. RESULTS

After quality filtering, an average of 2,767 sequences of the 16S rRNA gene was obtained per sample (range 790-6,550), which were assigned at the genus taxonomic level, giving a comparative view of the bacterial diversity and composition among teeth and gingival sites. A summary of these differences in composition can be observed in Figure 1.1. The top panels show the average bacterial proportions amplified for lingual and vestibular surfaces from the four quadrants, in teeth and gingival sulci. Pronounced differences were observed between vestibular and lingual sites of teeth and gingival sulci, indicating that some bacteria are

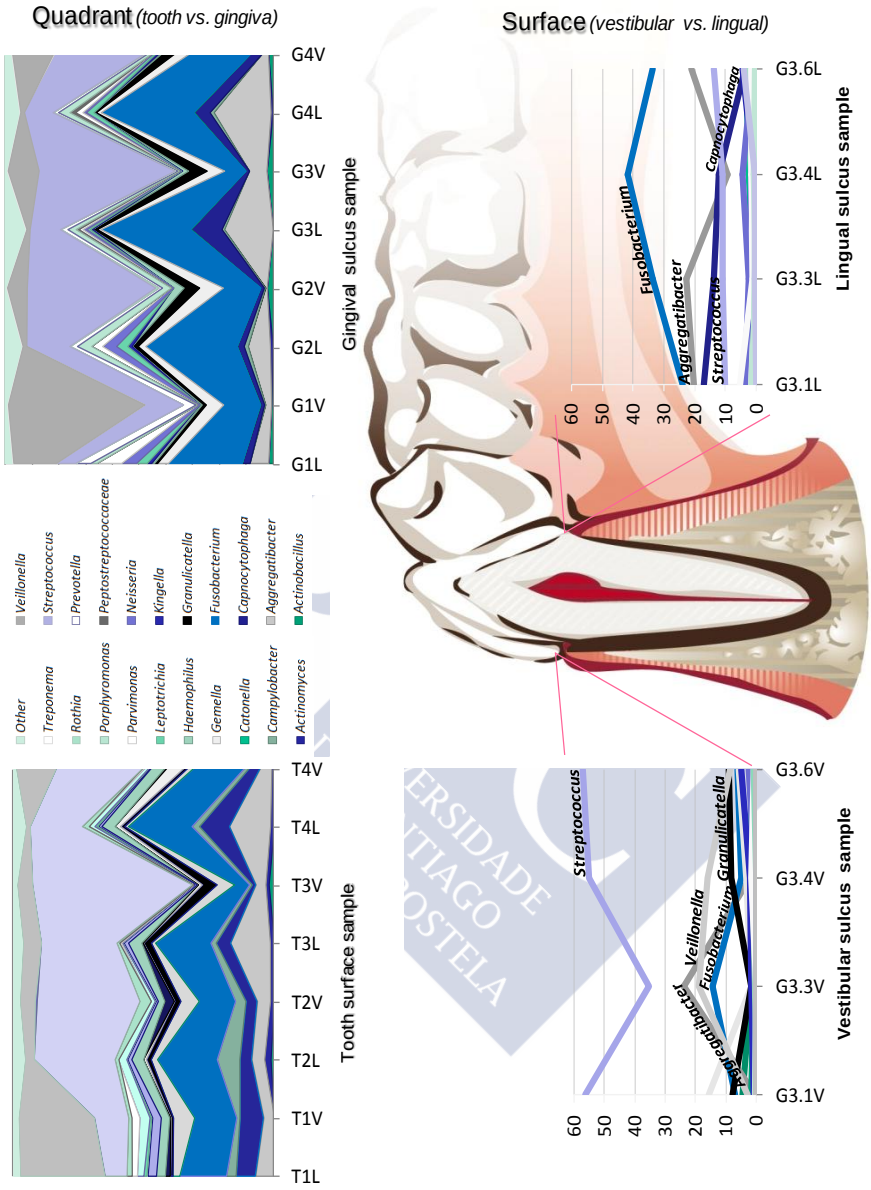

Figure 1.1. Bacterial composition across quadrants (top panels) and along the arcade within a given quadrant (lower panels). Top panels show the average proportion of bacteria at teeth surfaces (left graph) and gingival sites (right graph) at the four quadrants. The sample codes indicate whether teeth (T) or gingivae (G) were sampled, followed by the quadrant (numbers 1 through 4); V or L refers to vestibular (buccal) and lingual (palatine) surfaces, respectively. Lower panels show the variation in gingival bacterial composition for quadrant 3 at vestibular (left) and lingual (right) sites. First number at each sample refers to the quadrant and second number to the tooth, including first incisor (tooth 1), canine (tooth 3), first premolar (tooth 4) and first molar (teeth 6). V= vestibular; L= lingual.

characteristic for certain sites. For instance, genera like *Streptococcus* were found at 29-70% and 23-57% on the vestibular surfaces of teeth and sulci, respectively, but were found at lower levels on the lingual surfaces of the same tooth (0-51%) or sulcus (5-21%) in 97% of the samples. At both teeth and gingival sulci, the third and fourth quadrants had a more similar composition than quadrants one and two, and displayed a higher percentage of *Capnocytophaga* and *Aggregatibacter*. The difference in bacterial composition between the upper and lower arcades was significant for both gingival and teeth (Unifrac distance test with 1,000 permutations, $p<0.001$ in both vestibular and lingual comparisons).

Interestingly, when the compositional pattern along the arches was plotted separately for gingival samples from the vestibular and lingual sites (Figure 1.1 , lower panels), inverse relationships could be found for several bacteria, mainly involving *Streptococcus*, which appeared to increase in proportion when *Fusobacterium* and *Aggregatibacter* decreased, and vice versa. A similar pattern was found along teeth (Figure 1.2). In both dental surfaces and gingival sulci *Veillonella* was present at much higher proportion in canines than in incisors, premolars or molars. In most quadrants, *Streptococcus* was found at higher proportions in vestibular compared to lingual surfaces.

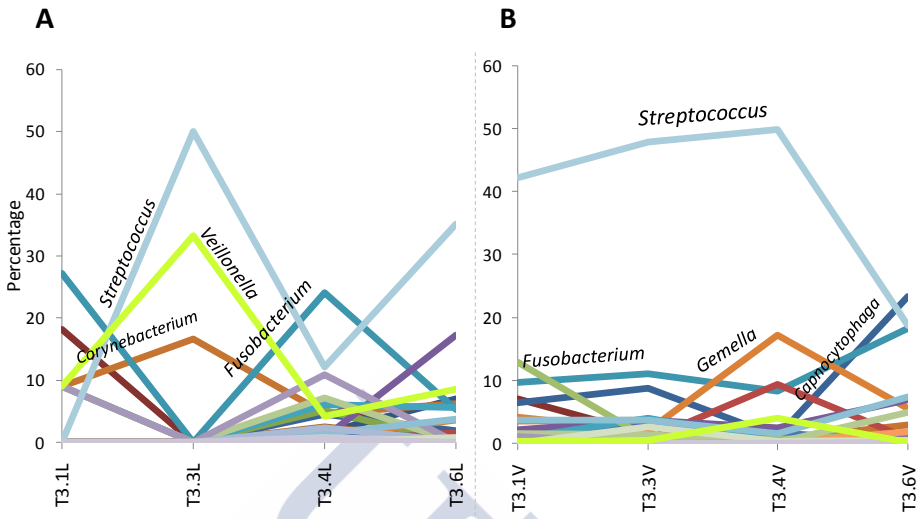

Figure 1.2. Composition of bacteria on tooth surfaces along the arcade, as estimated by pyrosequencing of the 16S rRNA gene. Graphs show the variation in dental bacterial composition for quadrant 3 at lingual (A) and vestibular (B) sites. The first number at each sample code refers to the quadrant and the second number to the tooth, including first incisor (tooth 1), canine (tooth 3), first premolar (tooth 4), and first molar (teeth 6). V= vestibular/buccal; L= lingual/palatine. Data correspond to individual MG01.

In addition to bacterial composition, diversity was also found to vary considerably at different tissues and sites within tissues. Sequences of the 16S rRNA were clustered at 97% nucleotide identity, which is considered the threshold for species boundaries (Yarza P et al., 2008). Thus, each cluster of sequences which are at least 97% similar forms an "Operational Taxonomic Unit" (OTU) that serves to estimate the approximate number of bacterial species. Saliva and tongue samples appeared to harbor a considerably higher number of OTUs than teeth or gingival sulci (Figure 1.3). Teeth and gingival samples had a similar level of diversity, and the lingual surface of gingival sulci showed the lowest level of OTUs.

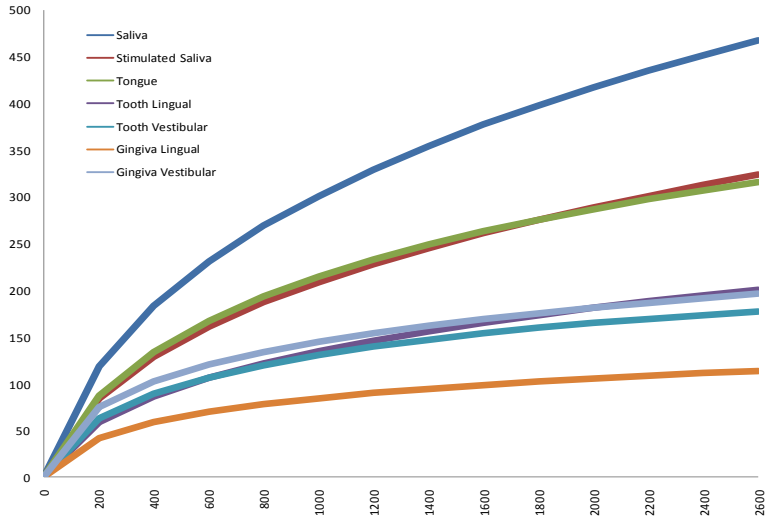

Figure 1.3. Bacterial diversity in different samples from individual MG01. Rarefaction curves indicate the relationship between sequencing effort and the estimated number of species-level OTUs (reads clustered at 97% sequence similarity). Saliva and tongue show the largest diversity. A similar result was found for individual MG02 except for saliva samples, with the stimulated saliva showing larger diversity than the non-stimulated saliva.

A convenient way to view the similarities among all samples can be provided by a Principal Coordinates Analysis (PCoA), where samples are closer or further from each other in a multidimensional space depending on their degree of similarity in bacterial composition. When applied to all samples, the PCoA showed a clear distribution pattern where gingival and dental samples cluster separately (Figure 1.4a). In addition, vestibular and lingual samples also appeared to cluster together with their own type, showing that each of these niches have a distinct bacterial composition.

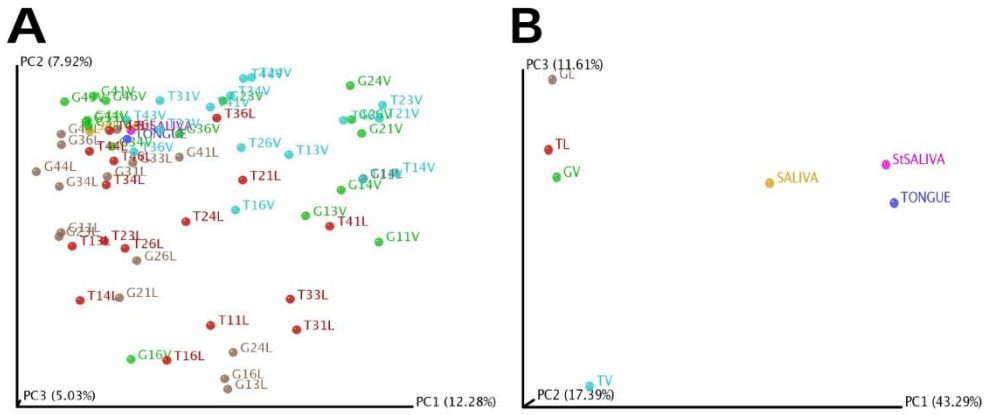

Figure 1.4. Relationship between gingival and dental samples in terms of bacterial composition. The Principal Coordinates Analysis (PCoA) was performed with Unifrac (Lozupone et al, 2006) and clusters individual samples according to species-level taxonomic composition (a). Saliva and tongue samples are included for comparison in a second PCoA in which dental (T) and gingival (G) samples from vestibular (V) and lingual (L) sites were pooled (b). Data are for individual MG01. A similar pattern was found in the PCoA for individual MG02.

Saliva samples taken from the two individuals showed a higher level of diversity compared to the vestibular and lingual areas of both teeth and gingival sites, suggesting that saliva must contain bacteria from other oral niches (Figure 1.3). Stimulated saliva showed a lower diversity than non-stimulated saliva in individual MG01 (Figure 1.3), but the reverse trend was observed in individual MG02. The latter would be consistent with the removal of bacteria from different oral cavity sites by the mechanical process of paraffin gum chewing. However, the PCoA plot showed that the stimulated saliva sample clustered tightly with the tongue sample (Figure 1.4b). This was confirmed by the comparative analysis of bacterial composition in saliva, tooth, gingival sulcus and tongue, which showed a remarkable similarity between tongue dorsum and stimulated saliva (Figure 1.5).

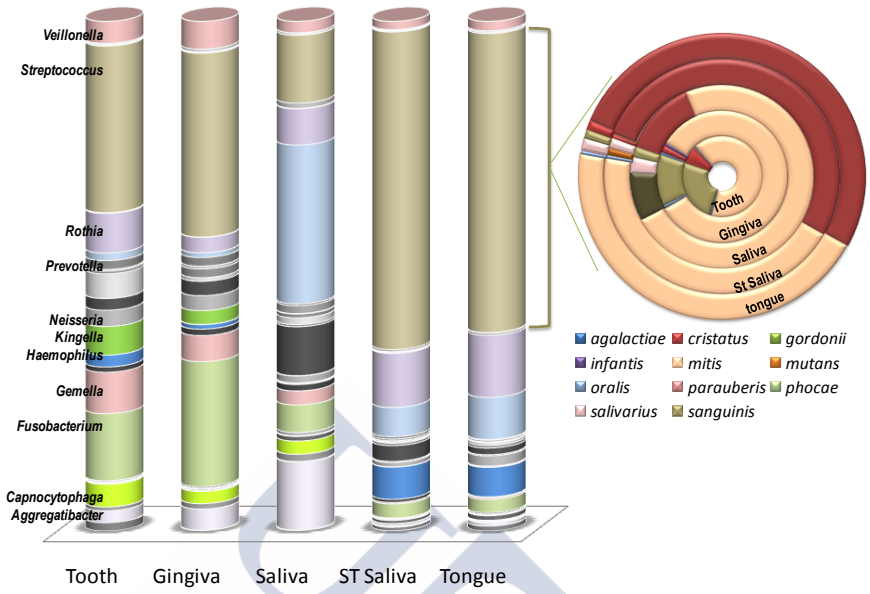

Figure 1.5. Bacterial composition at the genus level in different oral samples. Columns show the proportion of 16S rRNA sequences obtained from dental, gingival, unstimulated saliva, stimulated saliva and tongue from individual MG01. Streptococci are the dominant bacteria in most samples and a ring graph with the species assignments for this genus is shown on the right.

The PCoA plots showed that non-stimulated saliva appeared to have a very different microbial composition from that of teeth and gingival samples (Figure 1.4). The reason for this lack of correlation can be observed in Figure 1.5. Some of the most common bacteria in 24h supra- and sub-gingival plaque (particularly *Fusobacterium* and *Veillonella*) were at very low or even absent proportions in saliva; in addition, saliva also showed several bacterial genera at considerable proportions (including *Prevotella*, *Rothia* and *Haemophilus)* which were at very low levels or even virtually absent on teeth and/or gingival sites. This indicates that some bacteria colonizing the tooth and gingival sulcus are not represented in saliva and those microorganisms from oral sites other than the teeth or gingival are present in saliva at considerable proportions.

1.5. DISCUSSION

The data presented in the current manuscript demonstrate considerable differences in bacterial diversity and composition between individual sites and surfaces of the oral cavity, underlining the extraordinarily diverse set of microniches even within the same anatomical location. Particularly striking were the contrasts in bacterial composition between lingual and vestibular sites, as the proportion of some ubiquitous genera were highly characteristic of the surface sampled (Figures 1.1 and 1.2). For instance, *Streptococcus* was found on average at 48.2% and 43.8% in vestibular surfaces of teeth and gingival sulci, respectively, whereas it was detected at 23.9% and 12.33% at lingual surfaces. Thus, microbial diversity studies may be strongly biased if only one tooth surface or gingival site is sampled.

The reasons for such variability in microbial composition probably lie at the gradients and variation in physicochemical features at different locations of the mouth (Kleinberg I and Jenkins GN, 1964). One of these environmental factors affecting bacterial distribution could be oxygen. This is supported by the high proportion of obligate anaerobes like *Fusobacterium* at lingual sites, whereas aerobes or facultative anaerobes like streptococci are highly common at vestibular sites or incisors and canines, which are likely to be exposed to higher levels of oxygen. It is also very likely that pH plays a major role in that distribution pattern, as lingual and vestibular surfaces may have a different buffering effect of saliva. However, the opposite patterns displayed for some bacteria in the lower panel curves in Figure 1.1 also suggest possible antagonistic effects, which have been supported experimentally in the pairs *Fusobacterium-Streptococcus* (He H et al., 2012) and *Aggregatibacter-Streptococcus* (Whitmore SE and Lamont RJ, 2011).

We believe that this intra-oral variability in microbial colonization patterns should be taken into account when pooling dental plaque samples to assess bacterial composition. In addition, the fascinating site-specificity of bacteria observed in the present manuscript may have important implications for oral microbiological studies. Neither stimulated nor non-stimulated saliva were representative of the bacterial diversity, composition and proportions found at supra- and sub-gingival sites in the two individuals sampled in this study. Given that the tooth surfaces and the periodontal pockets are the sites where dental caries and periodontal disease take place, the use of saliva as a proxy of bacterial composition at those sites may not provide meaningful correlations between bacterial composition and disease status in epidemiological and etiological studies. Although some evidence has been found between microbial composition of saliva and oral diseases (Yang F et al., 2012), a major limitation is that most molecular studies performed thus far are association studies and the suggested biomarkers have not been tested in a longitudinal clinical study. Therefore, we do not know at present if the observed biomarker candidates are clinically relevant, and neither tests targeting single salivary bacteria nor salivary bacterial tests in combination with clinical parameters have been able to adequately predict the course of caries *in vivo* (Tellez M et al., 2013). In addition, some of these studies have found an association between microbiota and disease when using plaque samples but not saliva, both in periodontitis and dental caries (Huang et al, 2011; Ling Z et al., 2010), suggesting that saliva may not be representative of the microbial population at the disease site. Carious cavities, for instance, have been found to harbor a different set of bacterial species from dental plaque at sound surfaces, and the bacterial composition varies between enamel, dentin and deep dentin samples (Aas JA et al., 2008; Belda-Ferre P et al., 2012). Thus, including individuals with caries lesions at different stages of disease progression may introduce further noise in the detection of salivary biomarkers for caries. A further complication is given by

the use of different saliva sampling protocols in different laboratories, including paper points, mouth rinses and oral swabs (Kejser BJF et al., 2008; HMP, 2012).

Exploring microbial diversity in the oral cavity offers the possibility for a better understanding of the role of microorganisms in health and disease (Takahashi N and Nyvad B, 2012). However, precise and appropriate sampling must be made in order to relate health and disease status to microbial profiles. The work presented here is based on data from two individuals and studies with larger sample size would be needed to confirm the validity of the observed results. The variability of microbial diversity depending on the oral site sampled is nevertheless clear and we hope that the present study will help to decide the appropriate sampling strategy in future oral microbiological studies. Thus, I have decided to use samples from supragingival dental plaque and caries lesions in Chapters 2 and 3 of the current Thesis, as based on the results from this Chapter, those kind of samples would be expected to be more informative than saliva for studying dental caries etiology.

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A Tissue-dependent Hypothesis of Dental Caries

Simón-Soro A, Belda-Ferre P, Cabrera-Rubio R, Alcaraz LD, Mira A. A tissue-dependent hypothesis of dental caries. Caries Res. 2013;47(6):591-600.

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2.1. ABSTRACT

Current understanding of dental caries considers this disease a demineralization of the tooth tissues due to the acid produced by sugar-fermenting microorganisms. Thus, caries is considered a diet- and pH-dependent process. We present here the first metagenomic analysis of the bacterial communities present at different stages of caries development, with the aim of determining whether the bacterial composition and biochemical profile are specific to the tissue affected. The data show that microbial composition at the initial, enamel-affecting stage of caries is significantly different from that found at subsequent stages, as well as from dental plaque of sound teeth surfaces. Although the relative proportion of *Streptococcus mutans* increased from 0.12% in dental plaque to 0.72% in enamel caries, *Streptococcus mitis* and *Streptococcus sanguinis* were the dominant streptococci in these lesions. The functional profile of caries-associated bacterial communities indicate that genes involved in acid-stress tolerance and dietary sugar-fermentation are over-represented only at the initial stage (enamel caries), whereas other genes coding for osmotic stress tolerance as well as collagenases and other proteases enabling dentin degradation are significantly over-represented in dentin cavities. The results support a scenario in which pH and diet are determinants of the disease during the degradation of enamel, but in dentin caries lesions, not only acidogenic but also proteolytic bacteria are involved. We propose that caries disease is a process of varying etiology, in which acid-producing bacteria are the vehicle to penetrate enamel and allow dentin degrading microorganisms to expand the cavity.

2.2. INTRODUCTION

Dental caries is a multi-factorial disease caused by microbes and influenced by diet, hygiene habits, teeth shape and strength, saliva buffering capacity and host immune system, among others (ten Cate, 2009). Cavities are produced by demineralization of the teeth due to low pH, when the mineral part of the tissue dissolves due to desaturation, which is moderated by acids (Fejerskov O and Manji F, 1990). This would be possible when bacteria accumulate in dental plaque and ferment dietary carbohydrates over a sufficient amount of time, so the acid produced locally cannot be neutralized by the buffering capacity of saliva (Marsh PD, 1994). Although the ability of pH lower than a critical value to demineralize enamel is well established (Weatherell JA et al., 1984; Dawes C, 2003), it can only partially explain dentin caries because the mineral component is only a limited portion of dentin tissue. In fact, the two tissues are so different in nature, composition, structure and access to dietary food that explaining caries formation and progression by a single cause may not be feasible. In addition, the etiological factors of dental caries are also unclear. Although initial work related the presence of acidogenic mutans streptococci to dental caries (Loesche WJ et al., 1975; Loesche WJ, 1992) other microorganisms like lactobacilli or *Candida* spp have been proposed to produce acid and contribute to the disease (Becker MR et al., 2002; Munson MA et al., 2004). This gave rise to the non-specific plaque hypothesis, which sustains that caries can be the result of different acid-producing organisms (Theilade E, 1986). Important evidence showing that the disease is polymicrobial and would therefore not follow the classical Koch's postulates of infectious diseases has recently been provided by molecular methods, showing that mutans streptococci cannot be PCR-amplified or hybridize against specific DNA probes in a considerable proportion of cavities (Corby PM et al., 2005; Aas JA et al., 2008). These studies have shown that cavities can be complex ecosystems and suggest

that other species like *Atopobium*, *Veillonella* or *Corynebacterium*, which are preferentially found in caries lesions (Belda-Ferre P et al., 2012), could also be cariogenic. In fact, it has been proposed that dental caries is not caused by a concrete set of organisms but by a change in the community composition due to external factors which would shift the mineralization balance towards a destructuring of the tooth tissue (Takahashi N and Nyvad B, 2011). In all cases, the cause of dental caries as a whole is considered to be the acid produced by fermentation of sugars ingested in the food. In the current manuscript, we study dental caries through a metagenomic approach, presenting data that questions this single-cause explanation of the disease. We aim to test the hypothesis that caries, from both from a hard tissue and a microbiological perspective may be considered as a two-step process, namely initiation/demineralisation of enamel followed by progression through dentin.

In order to shed light on the causes of dental caries, a complete assessment of microbial diversity composition and function must be performed at different stages of caries progression and culture-based methods have proven to be insufficient because a large portion of oral bacteria cannot be cultured by classical laboratory methods and growth media (Aas JA et al., 2005). Seminal work by Aas and collaborators used amplification and cloning of the 16S rRNA gene and subsequent Sanger sequencing to study the microbial diversity in white-spot lesions, dentin caries and advanced cavitated lesions (Aas JA et al., 2008). This pioneering work suggested that the microbial taxonomic composition could change during caries progression but the low number of sequences obtained by this method (averaging 53 sequences per sample) limits enormously the full description of the diversity in these niches, which can reach several hundred species (Belda-Ferre P et al., 2012). The arrival of second-generation sequencing techniques like Roche pyrosequencing now allows obtaining hundreds or thousands of 16S rRNA sequences per sample obviating

the cloning steps (Keijser BJ et al., 2008). We have applied this technology to carious samples affecting enamel, dentin, and deep dentin, in order to make a more complete description of the bacterial diversity of dental caries affecting different tissues. In addition, we have performed direct pyrosequencing of the total DNA from the bacterial community at these three stages, allowing a description of the gene functions encoded by the microorganisms present. This metagenomic approach provides the first functional description of the different bacterial populations and their potential role in caries initiation and progression.

2.3. MATERIALS AND METHODS

2.3.1. Sample Collection and DNA Extraction

Supragingival dental plaque and caries samples were obtained from 22 volunteers after signing an informed consent. Sampling procedure was approved by the Ethical Committee for Clinical Research from the DGSP-CSISP (Valencian Health Authority, Spain). Oral health status of each individual was evaluated by a dentist following Nyvad B et al. (2003), with nomenclature from the Oral Health Surveys from the WHO. Samples were taken with autoclaved spoon excavators, following Aas JA et al. (2008). Plaque material from all teeth surfaces from each individual was pooled. In volunteers with active caries, dental plaque samples were taken without touching cavities. In those cases, material from individual cavities was also extracted and kept separately. Caries samples were classified as enamel caries (including “white spot lesions”), dentin caries and deep dentin caries (Aas JA et al., 2008). All samples were from active caries, as evaluated by their texture and colour (Nyvad B et al., 2003). All dentin caries samples were open and deep dentin caries samples selected did not reach the pulp; the absence of pulp exposure was evaluated radiographically, and an absence of

apical radiological area was observed for all samples. Volunteers were asked not to brush their teeth 24 hours prior to the sampling. Information was obtained regarding oral hygiene, diet and signs of periodontal disease. Complete information on sample size, teeth sampled and donors' clinical data is included in Table 2.1. DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison WI, USA), following the manufacturer instructions, adding a lysozyme treatment (1 mg/ml, 37ºC, 30 minutes). All 22 samples were used for PCR amplification and pyrosequencing of the 16S gene. In addition, three representative samples from enamel caries, dentin caries and deep-dentin caries were selected for direct, metagenomic pyrosequencing, based on radiographic evaluation and optimal DNA concentration and quality. The sequencing was performed at the Center for Advanced Research for Public Health using the GS-FLX sequencer (Roche, Basel, Switzerland) with Titanium chemistry, using ¼ of a sequencing plate per sample. Sequences from the three metagenomes were deposited at the NCBI Sequence Read Archive under accession number CA_05_4.6: 4447970.3, CA_06_1.6: 4447971.3, CA017_WS: 4450726.3.

Table 2.1. Clinical data of the studied samples. The number at the end of each sample code corresponds to the tooth from which the active caries sample was extracted. "P" indicates a dental plaque sample and "WS" indicates that several white spot lesions from the same individual were pooled.

SAMPLE	GENDER	AGE	IHO[1]	GINGIVAL INDEX[2]	BRUSH[3]	DMFT[4]			SEVERITY[5]
						DECAYED	*MISSING*	*FILLED*	
CA.001 3.8	H	51	3	2	0	5	11	0	4
CA.002 2.5	M	61	2	2	0	2	15	3	2
CA.005 4.7	M	42	2	1	1	7	2	1	3
CA.005 1.6	M	42	2	1	1	7	2	1	3
CA.006 2.2	M	49	3	3	0	6	2	0	4
CA.006 4.5	M	49	3	3	0	6	2	0	4
CA.007 4.3	M	45	3	2	0	9	13	0	4
CA.009 P	M	28	0	0	2	3	3	2	0
CA.011 1.2	M	24	0	1	1	17	0	0	3
CA.011 2.6	M	24	0	1	1	17	0	0	4
CA.011 P	M	24	0	1	1	17	0	0	0
CA.011 4.6	M	24	0	1	1	17	0	0	3
CA.013 P	H	21	3	3	0	21	0	0	0
CA.013 2.3	H	21	3	3	0	21	0	0	4
CA.013 2.1	H	21	3	3	0	21	0	0	3
CA.013 1.3	H	21	3	3	0	21	0	0	4
CA.017 WS	H	18	0	1	2	17	0	5	1
CA.017 P	H	18	0	1	2	17	0	5	0
CA1.01 1.2	H	38	1	1	1	5	0	14	1
CA1.01 2.3	H	38	1	1	1	5	0	14	1
CA.021 WS	M	25	0	0	3	8	0	8	1
CA.022 1.3	M	27	1	1	3	2	5	2	1

[1] Oral Health Index (OHI) values correspond to: 0= no plaque is observed; 1= plaque adheres to the probe; 2= plaque is observed in the gum region; 3= plaque is observed in 2/3 of the tooth.

[2] Gingival Index values correspond to: 0= healthy gingiva; 1= mild inflammation; 2= induced bleeding; 3= spontaneous bleeding. Brushing habits are indicated by the number of tooth brushes per day.

[3] Brush: number of toothbrushing per day. It corresponds to 0, 1, 2 and 3 times per day.

[4] DMF index values are separately shown, indicating the number of Decayed, Missing and Filled teeth.

[5] Caries severity values correspond to: 1= initial Caries, no clinically detectable loss of substance; 2= enamel caries, demonstrable loss of tooth substance in pits, fissures, or on smooth surfaces, but no softened floor or wall or undermined enamel; 3= dentin caries, detectably softened yellowish floor, undermined enamel, or a softened wall; 4= deep dentin caries, no bleeding observed when taking sample, lack of pulp exposure, absence of apical radiological area.

2.3.2. PCR Amplification and Pyrosequencing

The first 500 bp of the 16S rRNA genes were amplified in the 22 samples with the universal bacterial degenerate primers 27F and 533R using the high-fidelity AB-Gene DNA polymerase (Thermo Scientific, Waltham, MA, USA) with an annealing temperature of 52 ºC and 20 cycles to minimize PCR biases (Sipos R et al., 2007). The primers were modified to contain the pyrosequencing adaptors A and B and an 8bp "barcode" specific to each sample (McKenna P et al., 2008). Barcodes were different from each other in at least 3 nucleotides to avoid errors in sample assignments. Three PCRs were performed per sample, pooling their PCR products before purification, which was done using the Nucleofast PCR purification kit (Macherey-Nagel, Düren, Germany). The final DNA per sample was measured by picogreen fluorescence in a Modulus 9200 fluorimeter from Turner Biosystems (Sunnyvale, CA, USA) and the PCR products mixed in equimolar amounts. PCR products were pyrosequenced from the forward primer end only using a GS-FLX sequencer with Titanium chemistry (Roche, Basel, Switzerland) at the Center for Public Health Research (CSISP) in Valencia, Spain. One eighth of a plate was used for each pool of 20 samples.

2.3.3. Sequence Analysis

Reads with an average quality value lower than 20 and/or with more than 4 ambiguities in homopolymeric regions in the first 360 flows were excluded from the analysis. Only reads longer than 200 bp were considered. The ends of the reads, which consistently showed low quality values, were removed. Sequences were assigned to each sample by the 8-bp barcode and passed through the Ribosomal Database Project classifier (Cole JR et al., 2009); where each read was assigned a phylum, class, family and genus, as long as the taxonomic assignment was unambiguous within an 80% confidence threshold. To estimate total diversity, sequences were clustered at 97, 95 and 90%

nucleotide identity over a 90% sequence alignment length using the RDP pyrosequencing pipeline. For this analysis, sequences over 97% identical were considered to correspond to the same operational taxonomic unit (OTUs), representing a group of reads, which presumably belong to the same species (Yarza P et al., 2008). Principal Coordinates Analysis (PCoA) was performed with UNIFRAC (Lozupone CA et al., 2006) with clustering at 97% and the weighed option. This phylogenetic approach takes into account both taxonomically assigned and unassigned reads, using the distance between phylogenetic trees of the 16S gene to construct a matrix for the PCoA. Two-way statistical comparisons in bacterial composition were performed using the Unifrac metric (Lozupone CA et al., 2006) with 500 permutations and the Bonferroni correction. The DNA sequences were deposited in the MG-RAST server database with access numbers 4486962.3 through 4486985.3, under the project name "Caries Microbiome".

For the analysis of the three metagenomes, sequences were uploaded to the MGRAST server (Meyer F et al., 2008) and the functional assignment was retrieved at different hierarchical levels, making a table with the counts of reads per functional category and a normalized heatmap as explained in Belda-Ferre P et al. (2012). The over-representation of functions was estimated by the False Discovery Rate method applied to metagenomic sequences by the algorithm of White JR et al. (2009), testing the amount of false positive predictions (*q*-values) for a given *p*-value of significance. Metagenomic recruitment plots were performed using Nucmer and Promer v3.06, with default parameters (Kurtz S et al., 2004) and most abundant bacteria ranked according to the plots coverage (Belda-Ferre P et al., 2011).

2.4. RESULTS

2.4.1. Bacterial Diversity in Dental Plaque and Carious Lesions

An average of 1,882 16S rRNA high-quality reads were obtained per sample and between 8,000-12,000 per sample type, allowing a fairly complete description of bacterial diversity in dental plaque samples of unaffected teeth and in carious lesions affecting different tissues. When the number of 16S rRNA reads analyzed is plotted against the number of species-level phylotypes, a rarefaction curve is obtained in which the sampling effort is related to bacterial diversity. These curves show a dramatic decrease in bacterial diversity of all caries samples relative to the intact enamel surfaces from individuals with caries (Figure 2.1). Specifically, dental plaque samples from intact enamel reached 1,015 species-level Operational Taxonomic Units (an approximation of the number of species based on the 16S rRNA sequences, hereafter referred as OTUs) whereas white spots lesions affecting enamel had only 193 OTUs. These lesions showed the lowest level of diversity of caries samples, with dentin and deep dentin caries samples attaining 350 and 290 OTUs respectively. The higher microbial diversity of dental plaque suggests that bacteria inhabiting carious environments are a small subset of the bacterial community from intact enamel and that cavities represent selective niches which only specialized bacteria are able to colonize and exploit. The fact that the lowest level of diversity is found in enamel lesions indicates that this is the most stringent niche in human teeth, suggesting that the acidic environment probably acts as the main selective force reducing the number of species able to thrive in enamel caries.

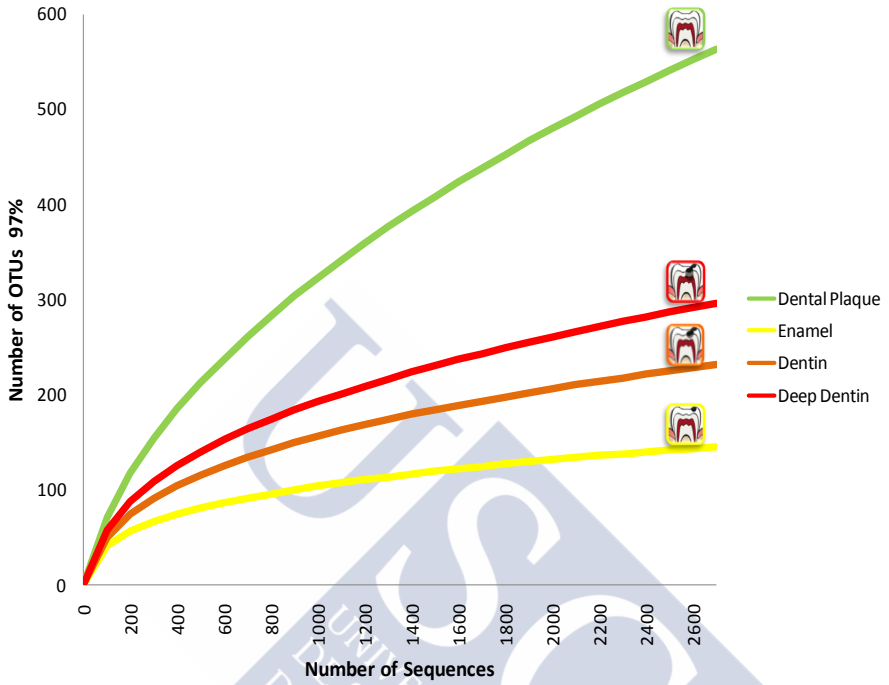

Figure 2.1. Bacterial diversity in dental plaque and caries lesions. The rarefaction curves showed indicate the relationship between sequencing effort and the estimated number of species in dental plaque samples, enamel caries, dentin caries and deep dentin caries lesions. The number of species-level phylotypes was calculated by pooling samples from the same sample type and clustering the sequences at 97% of sequence identity. This number of Operation Taxonomic Units (OTUs) is an estimate of species-level phylotypes. Carious lesions are between two and five times less diverse than dental plaque samples from sound teeth surfaces.

2.4.2. Bacterial Composition

Taxonomic assignment of the 16S rRNA reads indicated that the bacterial composition varied depending on the tissue affected. Dental plaque samples showed the highest number of assigned genera, which appeared to be more equally distributed than in carious samples, where some genera disappeared and others became more dominant (Figure 2.2a). In dentin caries, *Streptococcus* and *Prevotella* increased in proportion whereas *Neisseria*,

Capnocytophaga and *Fusobacterium* significantly decreased in relative numbers. Interestingly, *Lactobacillus* spp., which has been associated with dental caries because of their acidogenic potential, only appeared in deep dentin caries. This is in agreement with other reports indicating that *Lactobacillus* spp. doing not play a central role in caries initiation but in progression of the lesion (Shah AG et al., 2011). *Bifidobacterium* was detected at very low levels but although this could partly be due to the low amplification efficiency of this genus with universal primers (Hill JE et al., 2010).

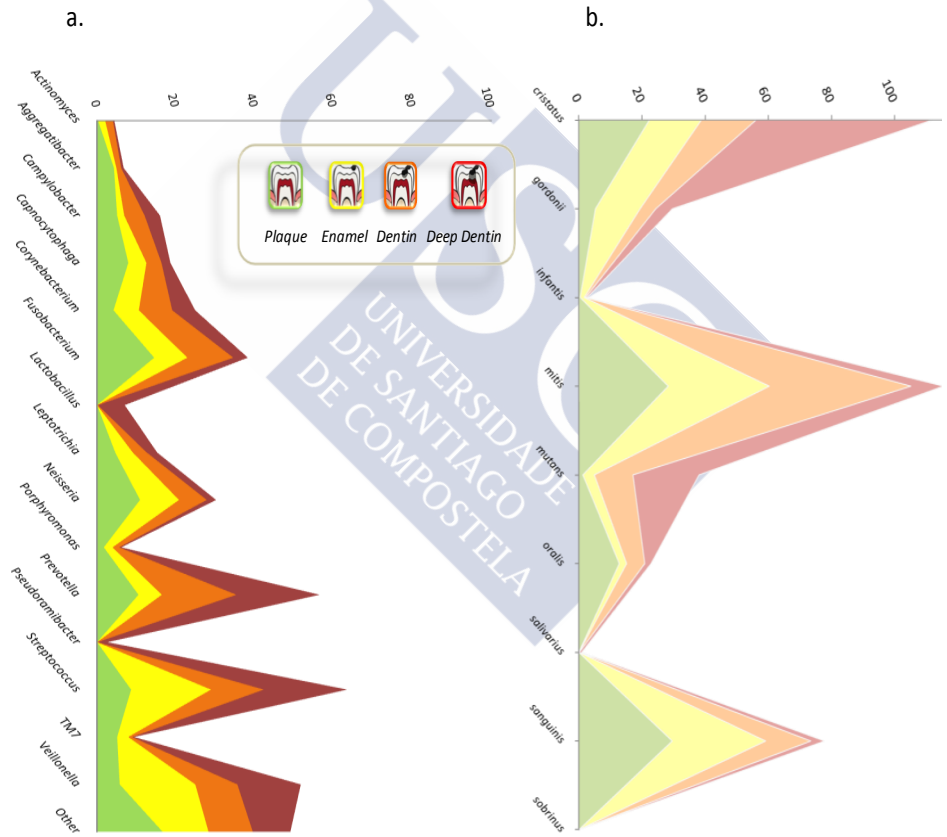

Figure 2.2. Bacterial composition in dental plaque and carious lesions. Graphs indicate (a) the taxonomic assignment at the genus level of PCR-amplified 16S rRNA pyrosequences in dental plaque samples, enamel caries, dentin caries and deep-dentin caries samples; and (b) the taxonomic assignment at the species level for streptococci. Several genera and *Streptococcus* spp. increase and others decrease in proportion as the disease progresses.

Metagenomic recruitment data also indicated a small contribution of this bacterium to the caries community. The increase in streptococcal species during caries progression was not due to mutans streptococci, which in fact appeared to diminish in dentin caries, as shown by the complete absence of recruitment of the *S. mutans* genome in dentin and deep-dentin cavities (Figure 2.3).

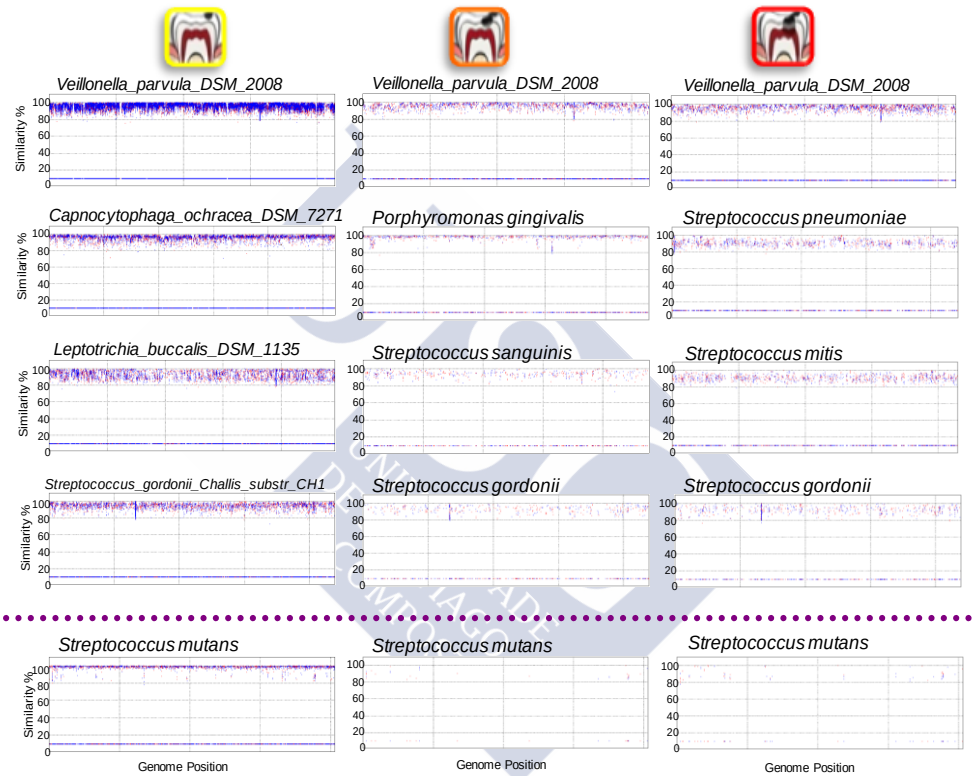

Figure 2.3. Most common species in enamel, dentin and deep dentin caries samples. Graphs are metagenomic recruitment plots in which each metagenomic read was compared against the genomes of all sequenced bacterial species available. Values on the Y axis indicate the sequence identity between each pyrosequence and the genome sequence of the 4 species with highest coverage, as well as for the species *Streptococcus mutans*. The coverage level is related to the proportion of DNA of that species in the sample and it is not subject to PCR amplification biases. The classic cariogenic species *S. mutans* is only detected in enamel caries lesions.

However, some proteolytic bacteria like *Prevotella*, which increase in proportion in dentin cavities, have been shown to be saccharolytic and tolerant to the acidic environment (Takahashi N et al., 1997) and could also be acidogenic (Aamdal-Scheie A et al., 1996). Although the assignment of streptococcal species using partial 16S gene sequences has to be taken with care, *S. mitis* appeared to be the dominant member of this genus in dentin cavities (Figure 2.2b). Strains most similar to *S. cristatus* appeared to increase in deep dentin cavities whereas *S. gordonii* and *S. mutans* increased in enamel caries compared to dental plaque from sound teeth surfaces (Figure 2.2b). Thus, the role of multiple organisms other than mutans streptococci for caries initiation and progression should be further studied. The metagenomic sequences obtained by direct pyrosequencing of total DNA allowed the search for fungal and protozoan genes. Genes from different *Candida* species (with best hits to *C. albicans*, *C. dubliniensis* and *C. tropicalis*) were found in enamel and dentin lesions. Given that this yeast has been related to an increase in caries incidence (Raja M et al., 2010), its presence at different stages of caries indicates that they may play a role in the disease. Although the relative presence of yeasts is very low when compared to the bacterial component of the community, these organisms are more resistant to standard cell lysis procedures than bacteria and therefore future work specifically aimed at studying fungal presence and diversity in caries lesions is required.

To investigate the similarity in bacterial composition among individual samples, a Principal Coordinates Analysis (PCoA) was performed. Although there was some degree of overlap between the different samples types, a clear pattern emerged in which caries lesions tended to cluster together with samples of the same kind (Figure 2.4). The bacterial composition of enamel caries was significantly different from those of dental plaque and dentin caries (Unifrac Distance, $p<0.002$ in both cases). The presence of a distinctive, tissue-dependent microbiota, suggests that the etiological factors of caries can be

different at different stages of caries progression, as it is confirmed by the data on gene function representation.

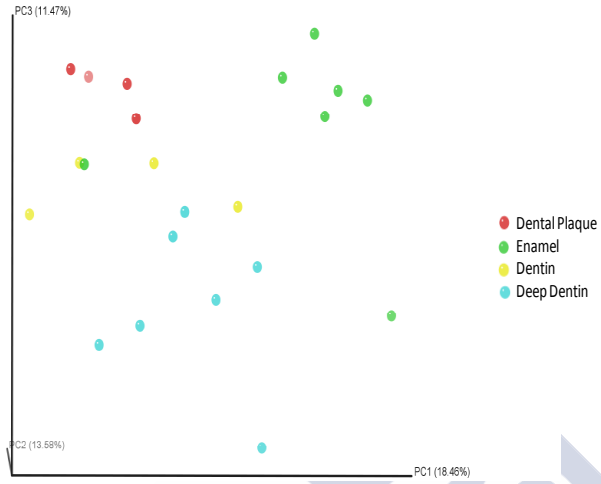

Figure 2.4. Variability in bacterial composition among samples. The Principal Coordinates Analysis (PCoA) plot separates samples according to caries status. Red dots represent dental plaque samples from sound teeth surfaces (n= 4); green dots correspond to enamel caries lesions (n= 7); yellow dots to dentin cavities (n= 4); and blue to deep dentin cavities (n= 7).

2.4.3. Functional Profile of Caries Progression

The second approach employed was a metagenomic study of the bacterial communities from white-spot lesions, dentin, and deep dentin lesions. We aimed to test the hypothesis that the functional profile of enamel and dentin caries communities is different. Functional assignment of 547,102 sequences totaling 171.87 Mbp from the total bacterial DNA against protein databases produced a functional profile which could be compared to that obtained by the same approach from dental plaque samples of individuals with and without caries (Belda-Ferre P et al., 2012). Using a clustering algorithm that groups samples according to the proportion of gene functions encoded in their DNA, dental plaque samples from individuals who had never suffered from dental caries clustered together, indicating that bacteria inhabiting the healthy teeth have a distinct set of genes. Dental plaque collected from healthy teeth in individuals with caries at the moment of sampling had a functional profile most

similar to that of white-spot lesions, whereas dentin lesions had a different profile from the rest (Figure 2.5). Thus, bacteria of caries samples affecting different tissues had a different functional profile.

Looking into detail at those functions over-represented in each sample type revealed the main features of the ecosystem and the microbial contribution to dental caries. As expected, enamel caries samples showed an over-representation of genes encoding complex carbohydrate fermentation, adhesion to cell surfaces and acid stress responses (q- and *p*-values of over-represented functions in enamel vs dentin metagenomes is shown in Table 2.2).

However, genes for dietary sugars fermentation and pH stress were at very low levels in dentin lesions, which showed instead an over-representation of genes for the metabolism of monosaccharides (q-value: 1.95×10^{-9}, $p=3.98 \times 10^{-11}$) and disaccharides (q-value: 3.58×10^{-21}, $p=2.43 \times 10^{-23}$), osmotic stress such as glycerol uptake proteins and aquaporins, adhesion to collagen and fibronectin, and a wide arsenal of peptidases, including collagenases and aminopeptidases. There is also an over-representation of enzymes which degrade human glycans and transport their sugar components like mannose (including mannose 6-phosphate isomerase, phosphomannomutase, and mannoside ABC transporters) and sialic acid (including a sialic acid transporter and a N-acetyl neuraminate synthase), and galactose (including galactokinase, α- and β-galactosidase), although the latter is also a dietary carbohydrate contained in lactate. In deep dentin samples, genes related to the immune response of the host were over-represented.

Figure 2.5. Functional profile of the microbiota present in dental plaque and cavities at different stages of caries progression. Each line represents a functional category, as assigned by the SEED system (Meyer F et al., 2008), depicted with a color indicating whether each function is over- or under-represented in the different samples. Each column corresponds to the data from individual samples of dental plaque from donors who never suffered from dental caries (teeth surrounded by a blue square), dental plaque of healthy teeth surfaces from donors with more than 10 cavities at the moment of sampling (teeth surrounded by a green square), enamel caries (white spot lesions), dentin and deep dentin samples. Statistically over-represented (OR) differences in relevant functions between enamel and dentin caries samples are indicated.

Table 2.2. Over-represented functions in the metagenome of enamel and dentin caries samples. p- and q-values are indicated for the comparison of frequencies between the two samples at the hierarchical level 2 of the SEED functional classification system (Meyer F et al., 2008), by an application of the FDR method modified for metagenomic data (White JR et al., 2009). Only statistically significant differences in gene functions are shown, which are color-coded for clarity. (Table continues next page)

Name	mean(ENAMEL)	mean(DENTIN)	pvalue	qvalue
Oxygen and light sensor PpaA-PpsR	0,00E+00	2.5464 e-05	4,27E-02	1,60E-01
Sulfate reduction-associated complexes	0,00E+00	2.5464 e-05	4,27E-02	1,60E-01
Bacterial cytostatics, differentiation factors and antibiotics	1,31E-04	1.993 e-05	3,23E-03	2,57E-02
Bacteriocins, ribosomally synthesized antibacterial peptides	1,46E-04	2,36E-04	4,21E-02	1,60E-01
SigmaB stress responce regulation	1,54E-04	2,95E-04	3,33E-02	1,41E-01
CBSS-316273.3.peg.922	1,78E-04	7.3631 e-05	1,52E-02	8,03E-02
Cyanate hydrolysis	1,78E-04	3,69E-04	1,94E-03	1,68E-02
Flavocytochrome C	1,81E-04	8.6363 e-05	3,58E-02	1,42E-01
Cold shock	1,81E-04	7.086 e-05	1,55E-02	8,03E-02
Aerotolerance operon in Bacteroides and potentially orthologous operons in other organisms	1,86E-04	5,16E-04	6.3932 e-08	2.0902 e-06
Phage shock protein (psp) operon	2,50E-04	1,01E-04	5,05E-03	3,66E-02
CBSS-159087.4.peg.2189	2,56E-04	9.9696 e-06	4.7785 e-08	1.7576 e-06
Muconate lactonizing enzyme family	3,15E-04	1,69E-04	1,90E-02	9,20E-02
Allantoin Utilization	3,70E-04	5,62E-04	1,56E-02	8,03E-02
Hfl operon	4,22E-04	6,14E-04	4,29E-02	1,60E-01
Ferrous iron transporter EfeUOB, low-pH-induced	4,34E-04	1,50E-04	5.9610 e-06	1,35E-04
Two-component regulatory systems in Campylobacter	4,40E-04	8,73E-04	1.7407 e-06	4.6565 e-05
Dissimilatory nitrite reductase	4,43E-04	2,67E-04	1,98E-02	9,38E-02
Adenosyl nucleosidases	5,48E-04	9,58E-04	4.8208 e-05	7,09E-04
Iron acquisition in Streptococcus	5,50E-04	7,31E-04	1,39E-02	7,56E-02
Acid stress	5,85E-04	2,50E-04	9.726 e-05	1,36E-03
Sporulation-associated proteins with broader functions	6,26E-04	3,56E-04	5,96E-03	3,90E-02
CBSS-211586.1.peg.3133	6,32E-04	1,10E-03	3.3697 e-05	5,22E-04
Flavohaemoglobin	6,58E-04	4,21E-04	2,82E-03	2,37E-02
Allophanate hydrolase 2 and Biotin carboxylase cluster	7,86E-04	5,24E-04	1,01E-02	5,81E-02
Biogenesis of c-type cytochromes	7,89E-04	1,12E-03	1,10E-03	1,08E-02
CBSS-316057.3.peg.3521	8,59E-04	6,28E-04	2,08E-02	9,56E-02
Proteorhodopsin	8,59E-04	5,87E-04	7,91E-03	4,85E-02
YjeE	1,09E-03	1,52E-03	4,14E-03	3,12E-02
Bacterial Chemotaxis	1,10E-03	1,78E-03	3.8655 e-07	1.1374 e-05
P uptake (cyanobacteria)	1,14E-03	1,61E-03	5,05E-04	5,50E-03
Heme, hemin uptake and utilization systems in GramNegatives	1,34E-03	1,02E-03	2,05E-02	9,56E-02
Stringent Response, (p)ppGpp metabolism	1,41E-03	1,02E-03	5,32E-03	3,66E-02
CBSS-320372.3.peg.6046	1,42E-03	1,77E-03	9,93E-03	5,81E-02
Murein Hydrolases	1,43E-03	1,76E-03	4,60E-02	1,65E-01
High affinity phosphate transporter and control of PHO regulon	1,44E-03	1,78E-03	2,92E-02	1,28E-01
CRISPs	1,44E-03	1,90E-03	2,95E-03	2,41E-02
Soluble cytochromes and functionally related electron carriers	1,53E-03	1,22E-03	3,45E-02	1,41E-01

Name	mean(ENAMEL)	mean(DENTIN)	pvalue	qvalue
Galactosylceramide and Sulfatide metabolism	1,76E-03	2,08E-03	2,25E-02	1,02E-01
Transposable elements	2,10E-03	2,87E-03	2.2363 e-05	3,87E-04
Gram-Positive cell wall components	2,20E-03	3,01E-03	8.6398 e-06	1,82E-04
Riboflavin, FMN, FAD	2,26E-03	2,61E-03	2,97E-02	1,29E-01
ECF class transporters	2,38E-03	3,08E-03	2,12E-04	2,65E-03
CBSS-281090.3.peg.464	2,39E-03	2,77E-03	4,93E-02	1,75E-01
Inorganic sulfur assimilation	2,68E-03	2,05E-03	3,39E-04	3,99E-03
Quinone cofactors	2,85E-03	2,27E-03	1,70E-03	1,61E-02
ZZ gjo need homes	3,11E-03	2,65E-03	3,39E-02	1,41E-01
Electron accepting reactions	3,30E-03	2,61E-03	1,87E-03	1,67E-02
Nitrate and nitrite ammonification	3,37E-03	2,89E-03	1,12E-02	6,33E-02
Coenzyme A	3,38E-03	3,00E-03	4,56E-02	1,65E-01
Biosynthesis of galactoglycans and related lipopolysacharides	3,54E-03	4,02E-03	3,97E-02	1,56E-01
Flagellar motility in Prokaryota	3,73E-03	5,88E-03	8.4156 e-18	8.2543 e-16
CBSS-196620.1.peg.2477	4,19E-03	3,70E-03	2,52E-02	1,12E-01
UDP-N-acetylmuramate from Fructose-6-phosphate Biosynthesis	4,36E-03	3,81E-03	4,36E-02	1,61E-01
Ton and Tol transport systems	4,66E-03	5,31E-03	3,42E-03	2,64E-02
Histidine Metabolism	4,73E-03	3,91E-03	4,85E-04	5,49E-03
NAD and NADP	5,38E-03	7,51E-03	1.4287 e-12	8.4083 e-11
Polysaccharides	5,40E-03	6,60E-03	1.1681 e-05	2,15E-04
Iron acquisition in Vibrio	5,40E-03	6,59E-03	4.6813 e-06	1,15E-04
Glutamine, glutamate, aspartate, asparagine; ammonia assimilation	6,26E-03	7,42E-03	1,84E-04	2,46E-03
Organic acids	7,16E-03	5,84E-03	9.3409 e-06	1,83E-04
Heat shock	7,58E-03	6,80E-03	1,26E-02	6,99E-02
Electron donating reactions	9,50E-03	8,71E-03	6,96E-03	4,45E-02
Arginine; urea cycle, polyamines	9,57E-03	8,84E-03	3,41E-02	1,41E-01
Branched-chain amino acids	1,12E-02	1,00E-02	8,48E-04	8,92E-03
Tetrapyrroles	1,28E-02	1,05E-02	1.4539 e-08	6.1118 e-07
Monosaccharides	1,40E-02	1,87E-02	2.4384 e-23	3.5875 e-21
Di- and oligosaccharides	1,66E-02	1,98E-02	3.9877 e-11	1.9556 e-09
Sugar utilization in Thermotogales	1,89E-02	2,30E-02	2.477 e-14	1.8221 e-12
Lysine, threonine, methionine, and cysteine	2,50E-02	2,36E-02	1,90E-02	9,20E-02
DNA repair	2,66E-02	2,86E-02	8,81E-04	8,94E-03
Phages, Prophages	2,75E-02	1,61E-02	6.0848 e-96	1.7901 e-93
Protein biosynthesis	6,78E-02	6,51E-02	5,69E-03	3,80E-02
Carbon storage regulator	1.1649 e-05	8.9727 e-05	2,16E-04	2,65E-03
carbazol degradation cluster	2.6212 e-05	1,01E-04	5,35E-03	3,66E-02
Bile hydrolysis related cluster	2.9124 e-06	4.9848 e-05	1,87E-03	1,67E-02
Pseudouridine catabolism	2.9124 e-06	9.0808 e-05	2.4406 e-05	3,99E-04
Iron Scavenging cluster in Thermus	2.9124 e-06	4.2641 e-05	7,61E-03	4,76E-02
Dessication stress	5.2424 e-05	0,00E+00	3,52E-02	1,42E-01
Tricarboxylate transport system	5.8249 e-05	1,62E-04	9,01E-03	5,41E-02
beta-glucuronide utilization	5.8249 e-06	3.9878 e-05	1,91E-02	9,20E-02
Sulfur oxidation	8.1549 e-05	1,93E-04	5,19E-03	3,66E-02
Phosphoenolpyruvate phosphomutase	8.7374 e-05	1,78E-04	1,73E-02	8,77E-02

These included the sortase *srt*A, which has been shown to have an anti-opsonization effect and be vital for bacterial colonization and infection in *S. sanguis* (Yamaguchi M et al., 2006), and genes for allantoin utilization. Allantoin stimulates cellular proliferation and helps to substitute necrotic for new tissues and must be at high levels at the pulp vascular tissues, exemplifying the degree to which deep-dentin bacteria have specialized in using nutrient sources that are available at that specific niche where ingested food may not reach, especially in hidden cavities. Iron-dependent siderophores are also over-represented in these samples, suggesting that iron must be a very scarce mineral within dentin.

2.5. DISCUSSION

2.5.1. A Microbial-Succession, Tissue-Dependent Hypothesis of Dental Caries

When we integrate the available information on caries microbiology with our own metagenomic data on caries lesions, dental caries emerges as a two-step process in which there is a succession of microorganisms. The complex microbial composition of cavities at different stages is clearly not consistent with the specific plaque hypothesis and supports a polymicrobial origin. *Streptococcus mutans* increases in proportion in caries lesions relative to dental plaque samples, but appears to be at very low frequencies, as previously reported (Gross EL et al., 2012). The microbial community undergoes a dramatic reduction in the number of species from a healthy to a diseased site (Figure 2.1). By considering the bacterial community within cavities, and looking at the predicted output of their activity, we have gained insights into the different ecosystems the carious process goes through. The functions those organisms encoded support a view in which enamel caries lesions are dominated by

acidogenic organisms which ferment dietary carbohydrates and where mutans streptococci are relatively common. This environment is acidic and enamel gets demineralized. However, when a channel is demineralized by their action and dentin is reached, the community changes significantly and is predominantly proteolytic, and the main environmental pressure has an osmotic nature. At this stage, the tissue has a mineralized component with a lower proportion of hydroxyapatite (HAP) and a higher critical pH which was estimated to be as high as 6.7 (Hoppenbrouwers PM et al., 1987), although lower values in the range 5.22-5.66 have been reported by recent *in vitro* work in root surfaces of human teeth (Shellis RP, 2010). In addition, pH values in dentin caries lesions appear to be variable (Hojo S et al, 1994). Thus, the low frequency of acid-stress and carbohydrate fermentation genes reported here could be influenced by the limited sample size of the study and should be confirmed in larger datasets. Although attempts to culture dentin bacteria with collagenolytic activity have not been successful, our metagenomic data show that collagenases are over-represented, especially in *Prevotella* species. The data suggest that dental caries cannot be considered a classical infectious disease (Fejerskov O, 2004) both in the sense that Koch's postulates are not applicable and in the sense that the microbial players involved change through time depending on the tissue affected and in the sense that multiple causes are responsible for the progressing lesion at the different stages of the caries process.

2.5.2. The Role of Bacterial vs. Host Proteolytic Enzymes in Dentinal Caries

The present study is the first metagenomic approach of caries lesions, in which the total DNA of the complex microbial community was sequenced and analyzed. This allowed us to characterize the main functions encoded in the microbial genomes of the caries dwellers, identifying the presence in dentin caries of abundant proteolytic enzymes, including bacterial collagenases, serine-

proteases, glycoproteases, carboxy-terminal proteases and metalloproteases, as shown in Table 2.2. Thus, we proposed that in addition to human metalloproteinases, the formidable microbial-encoded proteolytic arsenal may play a significant role in dentinal protein degradation. There is strong experimental evidence of collagenase activity in oral bacteria, ranging from Streptococcus spp. to Actinomyces, Enterococcusfaecalis, Porphyromonas spp., Prevotella spp, Capnocytophaga, Eubacterium, Bifidobacterium or Treponema, among others (Harrington D, 1996).

Human collagenolitic activity through the role of MMPs in the degradation of dentin matrix has been demonstrated both directly (Tjäderhane L et al., 1998a) and indirectly in animal experiments in which MMP inhibitors reduce dentin caries progression (Tjäderhane L et al., 1999; Sulkala M et al., 2001). Treatment of dentin with acidic adhesive monomers has been shown to activate dentinal MMPs (Mazzoni A et al., 2006; Nishitani Y et al., 2006). Experimental data with MMP inhibitors reducing dentin collagen matrix degradation - in the absence of bacteria - is also clear (Tjäderhane L et al., 2013a,b). High levels of MMP-8 and -9 in caries-infected compared to caries-affected dentin (Shimada Y et al., 2009) may indicate saliva as a source of these enzymes in dentinal caries. This is supported by the strong correlation of salivary MMP-8 with caries. Recently, collagenolytic cysteine cathepsins have also been identified in intact (Tersariol IL et al., 2010) and carious dentin (Nascimento FD et al., 2011). Saliva also contains cysteine cathepsins (Nascimento FD et al., 2011), at least cathepsin B (van Strijp AJ et al., 2003), and cathepsin inhibitors cystatin and lipocalin-1 are more abundant in saliva and pellicle of caries-free subjects than those with high DMFT (Vitorino R et al., 2006). MMP- and cysteine cathepsin activities are significantly higher in carious than in intact dentin (Nascimento FD et al., 2011; Vidal et al., in press). MMP-2 (Boushell LW et al., 2011), MMP-9 (Zehnder M et al., 2011) and MMP-20

(Sulkala M et al., 2002) are increased in dentinal tubules of carious teeth. MMP-2 expression is up-regulated in odontoblasts under the carious lesion (Charadram N et al., 2012). Furthermore, MMPs have also been recently indicated as susceptibility genes for dental caries (Tannure PN et al., 2012a,b; Wang et al., 2013). What we have reported is the presence of bacteria-encoded collagenases and other proteases in dentine cavities, which we propose must contribute to dentinary tissue degradation in addition to human MMPs.

Contrary to human MMPs, bacterial-encoded metalloproteinases are badly characterized and important efforts are needed to accurately classify and characterize those (Duarte et al., 2014). Partly due to this, the microbial protein classification systems do not include a functional category corresponding to bacterial collagenases. The p- and q-values shown in the Table 2.2 are indicated for the comparison of frequencies between dentine and enamel caries samples at the hierarchical level 2 of the SEED functional classification system (Meyer F et al., 2008). At this level, the difference in frequency for the Protein Degradation function to which collagenases belong is not significant, as there are other proteases which do not show significant differences between the two groups. However, when we go down to lower hierarchical functional annotation levels, there are significant differences for aminopeptidases C ($p=0.048$), alanine aminopeptidases N ($p<0.0001$), ATP-dependent clp proteases ($p=0.02$) or Xaa-Pro aminopeptidases ($p=0.0009$), strongly supporting that bacterial communities in dentin caries are highly proteolytic when compared to the enamel caries niche. When we focus on bacterial collagenases alone, there are 0.326 reads per Mbp in enamel caries samples vs 0.857 per Mbp in dentin caries, again suggesting collagenolytic potential in dentin microbes, which will have to be confirmed experimentally.

Over 20% of collagenases hits corresponded to Prevotella, which could therefore be a vital microbial component of dentin tissue degradation. Porphyromonas gingivalis, which is at low levels in dentinal lesions, has a more intense activity (Eley BM and Cox SW, 2003). However, we want to underline that the bacterial isolates with which these experiments were performed were cultured from periodontal samples and different species could be present in dentinal lesions. In fact, very few strains have been isolated from dentinal samples and efforts should be directed to culture microorganisms isolated from carious lesions in order to experimentally demonstrate/test their proteolytic potential. A few candidates are suggested by our data and are not limited to *Prevotella* spp., as several significant hits were obtained with maximum similarity to annotated collagenases from *Bacteroides*, *Campylobacter*, *Clostridium*, *Acinetobacter*, *Capnocytophaga*, *Treponema* or *Burkholderia*, among others.

As it has previously been acknowledged, the presence of a given microbial genetic repertoire as shown by their metagenomic DNA analysis does not prove their expression (Nyvad B et al., 2013). Thus, future work should be focused on RNA-based, meta-transcriptomic approaches where the sequencing of mRNAs could confirm the production of bacterial-encoded metalloproteinases. Metatranscriptomic approaches to study in vivo oral microbial communities have recently been performed in subgingival and supragingival dental plaque (Durán-Pinedo A et al., 2014, Benítez-Paez A et al., 2014) and several efforts are under way to study microbial and human gene expression in carious dentin. We propose bacterial proteases are the only source of dentin degradation, on the basis of the data shown; but we do want to emphasize that human metalloproteinases and cysteine cathepsins may not be the only proteases enabling dentinal caries progression. We hope that our discovery of bacterial-encoded metalloproteinases in dentin caries

metagenomes stimulates both the isolation and characterization of potentially proteolytic dentin bacteria and the analysis of microbial-encoded mRNAs from caries samples to ultimately quantify the contribution of microorganisms to the proteolysis of dentinary tissue.

2.5.3. Clinical Relevance

There are several clinical observations that can be explained in the light of the microbial hypothesis of dental caries. One of them is the funnel shape of enamel lesions. Teeth radiographs consistently show that the aperture on the external side of the enamel lesion is wider than that on the dentin side (Ekstrand K et al., 1991). If the internal pH in dentin lesions was also acidic, a double-funnel shape would be expected (Figure 2.6). However, radiographs of carious teeth rarely show a reduction of enamel width from the inside, indicating that the global output of the bacterial community in dentin caries lesions, although it contains both acidogenic and proteolytic organisms, may indeed be considerably less acidic than the enamel caries microbiota (Zaura E et al., 2002). Compared to enamel lesions, dentin cavities –particularly hidden or unexposed lesions- are expected to have lower availability of fermentable dietary carbohydrates and this could influence the acidogenicity of the environment. Nevertheless, due to the chemical properties of dentin HAP, critical pH is higher than that of enamel (Hoppenbrouwers PM et al., 1987) and tissue demineralization can proceed even in moderately acidic conditions. The consequences of proteolytic metabolism in dentin microorganisms should also be evaluated, as the by-products of proteolysis could in fact contribute to increase the pH. Proteolytic microbial activity in dentin has also been shown to induce production of human metalloproteases, which can further degrade the tissue (Chaussian-Miller C et al., 2006; van Strijp AJ et al., 2003).

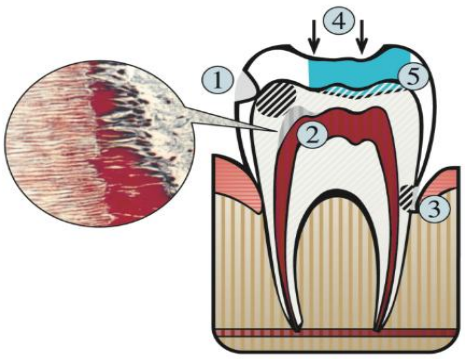

Figure 2.6. Clinical evidence of the tissue-dependent hypothesis of dental caries. The shape of enamel caries lesions has normally a funnel-like structure (1) – not a double-funnel shape – , and enamel is in most cases not degraded from the inside in dentin cavities; both observations indicate that the pH in dentin cavities is not as acid as on enamel lesions. In coronal caries, occlusal forces during mastication can break the enamel over dentin lesions due to lack of structural support (4) but the enamel is not degraded by the caries lesion itself. Caries can progress through the dentin even in inter-proximal lesions and under cavity restorations (5) where access to dietary carbohydrates is limited or even absent. In reactive dentin (2), the tissue is highly mineralized, but dentin-specialized bacteria are deprived of nutrients and unable to perform carbohydrate fermentation or progress through the obliterated dentinal tubules, which effectively stops the caries process. The known bacterial composition of root caries lesions where enamel is absent (3) resembles that of dentin cavities and not enamel caries lesions, again suggesting that the microbial community is tissue-dependent.

The progression of cavities is best seen in interproximal lesions because of the absence of occlusal forces and the limited availability of food remnants. In these circunstances, the dentin lesion expands radially and reaches the inside part of enamel, *without* degrading it, again suggesting a limited acidity unable to demineralise enamel. In coronal cavities however, masticatory forces can break the enamel located over the dentin cavity, giving the wrong impression that the enamel has been degraded by the caries when in fact it has been fractured by mastication (Figure 2.6). In the absence of enamel breakdown by mastication however, the caries mineral dissolution merely forms a funnel-shape channel through enamel to an entirely new niche with stringent anaerobic and osmotic characteristics, under different immunological challenges and where nutrient availability from food is limited. Physiological, morphological and genetic studies of bacterial isolates from dentin show some of their immune evasion mechanisms (Jontell et al., 1998), highlight an important ability to liberate sugars from human glycoproteins (Paddick JS et al., 2005) and capacity to adhere to dentinal tissue (Love RM et al., 1997), and show that the size and

shape of these bacteria allow them to proceed through the dentinal channels (Love RM and Jenkinson HF, 2002). Our metagenomic data also illustrate this formidable adaptation to survive in the dentinal tissue and anticipate fascinating mechanisms of nutrient utilization and interactions with the immune system. One of the pulp tissue's responses against bacterial invasion is the formation of reactive dentin. In this altered tissue the dentinal channels are irregularly arranged and blocked by formation of crystals, preventing the advancement of bacteria towards the pulp (Love RM and Jenkinson HF, 2002). In addition, the tissue is devoid from water and proteins, including collagen (Nanci A, 2008). If acidity was the only cause of caries progression, reactive dentine should be degraded by sugar-fermenting organisms but this modified tissue in fact reduces effectively the progression of the lesion. In the light of the microbial hypothesis presented here, dietary sugar-fermenting organisms are a minority in dentin caries and do not have access to fermentable complex carbohydrates, whereas dentin dwellers are also specialized in proteolytic digestion of dentinal tissue and would therefore not be able to create the acidogenic environment that presumably would be necessary to break reactive dentin.

Our data underline that a better accuracy in caries description would be desirable, as individuals with the same oral health indexes could vary considerably in the presence of cavities at different stages. An example is given by *Lactobacillus*, which we have found only representative in deep dentin caries. Thus, we believe that even if saliva sampling is found to be appropriate for epidemiological studies, etiological research must focus on individual lesions.

If caries disease is a tissue-dependent process, standard preventive measures such as dietary reduction of fermentable sugars (Marsh PD, 2003), pH regulation strategies (Stookey GK, 2008) and targeting of acidogenic species

for immunization (Abiko Y, 2000) would still represent valid approaches because they would be centered in reducing the initiation of cavities. However, caries progression would involve a different set of bacterial players and tissue-degrading processes, and is likely to be relatively independent of diet. Thus, buffering of acidity and diet control may have a reduced effect once the lesion has demineralized enamel and entered dentinal tissue. In addition, active and passive immunization strategies should not be aimed exclusively at one species, but use antigens which can target different bacteria involved in the caries process (Mira A et al., 2004; Mira A, 2007). Even within enamel caries, the diversity of bacteria is wide (Figure 2.1), and includes many potentially acidogenic bacteria outside mutans streptococci (Figure 2.3). Thus, our data do not support the specific plaque hypothesis (Loesche WJ et al., 1975; Loesche WJ, 1992) as it suggests that caries initiation and progression appears to be the result of microbial consortia. Further work should aim to determine whether those bacterial consortia are formed by specific bacteria or whether the acidogenic output responsible for enamel caries initiation can be produced by shifts in the bacterial community equilibrium regardless of their specific composition, as the Ecological Hypothesis has proposed (Marsh PD, 1991; Takahashi N and Nyvad B, 2011).

We hope that the current manuscript stimulates further metagenomic work on caries lesions, especially hidden cavities where contamination from non-dentin bacteria would not exist. Culturing the etiological agents of dentin caries could also proof fruitful as their proposed non-acidogenity, proteolytic and glycanolytic capabilities could be experimentally tested. In order to test cavities acidity, accurate *in vivo* pH measurements of dentine lesions, especially non-exposed lesions, are required, and recent advances in pH microscopic probes and pH-dependent dyes (Hiraishi N et al., 2003; Schlafer S et al., 2011) could make this possible in the near future. In addition, it has to be kept in mind that

DNA-based metagenomic approaches cannot distinguish live from quiescent or dead bacteria and future studies should focus on RNA-based or protein-based methodologies (metatranscriptomics and metaproteomics approaches, respectively) to focus on the role played by the active fraction of the oral microbiota (Nyvad B et al., 2013).

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3

Metatranscriptomics Reveals overall Active Bacterial Composition in Caries Lesions

Simón-Soro A, Guillen-Navarro M, Mira A. *Metatranscriptomics reveals overall active bacterial composition in caries lesions*. J Oral Microbiol. 2014 Oct 24;6:25443.

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3.1. ABSTRACT

Identifying the microbial species in caries lesions is instrumental to determine the etiology of dental caries. However, a significant proportion of bacteria in carious lesions have not been cultured, and the use of molecular methods has been limited to DNA-based approaches, which detect both active and inactive or dead microorganisms. The aim of this Chapter was to identify the RNA-based, metabolically active bacterial composition of caries lesions at different stages of disease progression in order to provide a list of potential etiological agents of tooth decay.

Non-cavitated enamel caries lesions (n= 15) and dentin caries lesions samples (n= 12) were collected from 13 individuals. RNA was extracted and cDNA constructed, which was used to amplify the 16S rRNA gene. The resulting 780 bp PCR products were pyrosequenced using Titanium-plus chemistry and the sequences obtained were used to determine the bacterial composition.

An average of 4,900 reads of the 16S rRNA gene was obtained per sample, giving a comprehensive view of the active bacterial communities in caries lesions. Estimates of bacterial diversity indicate that the microbiota of cavities is highly complex, each sample containing between 70 and 400 metabolically active species. The composition of these bacterial consortia varied among individuals and between caries lesions of the same individuals. In addition, enamel and dentin lesions had a different bacterial makeup. Lactobacilli were found almost exclusively on dentin cavities. Streptococci accounted for 40% of the total active community in enamel caries, and 20% in

dentin caries. However, *Streptococcus mutans* represented only 0.02-0.73% of the total bacterial community.

The data indicate that the etiology of dental caries is tissue dependent and that the disease has a clear polymicrobial origin. The low proportion of mutans streptococci detected confirms that they are a minority and questions its importance as the main etiological agent of tooth decay. Future experimental work should be performed to confirm the cariogenicity of the identified bacteria.

3.2. INTRODUCTION

It has been estimated that approximately 50% of oral bacteria have not been cultured to date (Aas JA et al., 2005). Classical studies based on microbial culture established mutans streptococci and lactobacilli as the main causative agents of dental caries (Loesche WJ et al., 1975; Badet C and Thebaud NB, 2008). However, other microbial species were also isolated from caries lesions and have been related to the disease, including bifidobacteria and *Scardovia* (Mantzourani M et al., 2009; Tanner AC et al., 2011). Furthermore, the application of molecular cloning and Sanger sequencing to study carious lesions at different stages of the disease revealed that although *S. mutans* levels correlated with disease severity, it could not be always amplified by PCR whereas other bacteria were present, including *Prevotella*, *Atopobium* and *Propionibacterium* (Aas JA et al., 2008). When the metagenomic DNA from individual dentin caries samples was directly sequenced without the need for PCR, the genus *Veillonella* appeared dominant within a surprisingly diverse community (Belda-Ferre P et al., 2012), underlining the varying nature of

microbial composition in cavities. The application of pyrosequencing to PCR products of the 16S rDNA gene has become an extremely powerful approach, revealing that cavities are extraordinarily diverse ecosystems (Gross EL et al., 2012) where *S. mutans* accounts at most for 1.6% of the carious lesion bacterial community (Simón-Soro A et al., 2013b).

A drawback of these DNA-based studies is that the PCR step may amplify DNA from inactive or even dead microorganisms, making it necessary to determine the functional bacteria that effectively contribute to the disease (Nyvad B et al., 2013). A way to achieve this is to perform the 16S gene amplification starting from RNA material, given that the amount of rRNA material in bacterial cells is known to be related to their degree of metabolic activity (Gentile G et al., 2006; Amann RI et al., 1995). In the current work, we have performed PCR amplification of RNA extracted from enamel and dentin caries lesions, after a reverse-transcription step (Benítez-Páez A et al., 2014). The obtained PCR products were then pyrosequenced with the aim of characterizing the active bacterial composition of cavities.

3.3. MATERIALS AND METHODS

3.3.1. Sample Collection

All donors signed a written informed consent and the sampling procedure was approved by the Ethics Committee from the DGSP-CSISP (Valencian Health Authority), with reference 10/11/2009. All donors attended the University of Santiago Dental Clinic, had not been treated with antibiotics or antifungals in the previous 6 months, had all 28 teeth present (excluding third

molars) and had not suffered from any systemic disease. Clinical data are shown in Table 3.1. All caries lesions sampled were active lesions as assessed by their texture and colour, and active white spot lesions were identified because they appear chalky white, opaque and rough (Nyvad B et al., 1999).

All enamel caries collected (n= 15) were non-cavitated ("white spot" lesions) and were collected with sterile spoon excavators. Supragingival dental plaque samples in caries-bearing individuals were taken 24 hours after toothbrushing from vestibular and palatine surfaces of the teeth using the same procedure in Chapter 1 (Simón-Soro A et al., 2013a). Teeth were not dried before sampling. Unstimulated saliva samples were collected by drooling, as previously described in the same Chapter (Simón-Soro A et al., 2013a). Open dentin caries lesions (n= 6) were sampled directly with a sterile spoon excavator, after removing the top, biofilm layer in contact with the oral environment. All carious teeth were isolated with rubber dam to reduce the risk of saliva contamination. Unexposed dentin cavities ("hidden dentin lesions", n= 6) were assessed radiographically, and a water-cooled diamond bur in an air-turbine handpiece was used to drill the enamel. Water circulation was stopped right before reaching dentinary tissue to minimize contamination, and the dentine lesion was hand excavated with a sterile spoon excavator. Radiographic images revealed no evidence of pulp necrosis in any dentin carious lesion sampled. Samples were eluted in 500 ul of PBS buffer and immediately frozen at -80 °C. RNA concentrations obtained ranged from 12-120 ug/ul. All samples were diluted to 12ug/ul before PCR was performed.

Table 3.1. Characteristics of the samples and individuals included in this study. First part (red) describes the features of caries lesions. Second part (pink) of the table includes clinical data of individuals.

Sample	*Lesion Code*	*Tooth*	*Severity*	*Location*	*Patient*	Gender	Age	OHI[1]	GI[2]	Toothbrush per day	Decayed	Missing	Filled
CA021.46WS	WS	4.6	1	V	CA.021	Woman	25	0	0	3	8	0	8
CA021.34WS	WS	3.4	1	V	CA.021	Woman	25	0	0	3	8	0	8
CA021.36WS	WS	3.6	1	V	CA.021	Woman	25	0	0	3	8	0	8
CA022.13WS	WS	1.3	1	V	CA.022	Woman	27	1	1	3	2	5	2
CA022.46WS	WS	4.6	1	V	CA.022	Woman	27	1	1	3	2	5	2
CA022.47WS	WS	4.7	2	V	CA.022	Woman	27	1	1	3	2	5	2
CA078.36WS	WS	3.6	1	V	CA.078	Woman	21	0	1	3	8	0	9
CA078.45WS	WS	4.5	1	V	CA.078	Woman	21	0	1	3	8	0	9
CA078.47WS	WS	4.7	1	V	CA.078	Woman	21	0	1	3	8	0	9
CA078.37WS	WS	3.7	1	V	CA.078	Woman	21	0	1	3	8	0	9
CA078.46WS	WS	4.6	2	V	CA.078	Woman	21	0	1	3	8	0	9
CA088.47WS	WS	4.7	1	V	CA.088	Woman	23	2	1	1	4	1	10
CA089.45WS	WS	4.5	1	V	CA.089	Woman	44	1	0	3	0	0	12
CA101.12WS	WS	1.2	1	V	CA1.01	Man	40	1	1	1	5	1	14
CA101.23WS	WS	2.3	1	V	CA1.01	Man	40	1	1	1	5	1	14
CA078.47H	H	4.7	3	I	CA.078	Woman	21	0	1	3	8	0	9
CA078.37H	H	3.7	3	I	CA.078	Woman	21	0	1	3	8	0	9
CA081.48H	H	4.8	3	V	CA.081	Man	40	2	2	1	3	1	3
CA083.27H	H	2.7	3	II	CA.083	Woman	79	0	1	2	4	2	8
CA085.47H	H	4.7	3	II	CA.085	Woman	36	0	0	2	1	1	10
CA086.28H	H	2.8	3	II	CA.086	Man	30	2	2	1	1	0	8
CA077.38DD	DD	3.8	3	II	CA.077	Woman	31	2	2	2	9	6	9
CA077.48DD	DD	4.8	3	II	CA.077	Woman	31	2	2	2	9	6	9
CA079.15DD	DD	1.4	3	II	CA.079	Man	17	1	1	2	2	0	1
CA079.21DD	DD	2.1	3	V	CA.079	Man	17	1	1	2	2	0	1
CA082.34DD	DD	3.4	3	II	CA.082	Woman	52	2	2	0	3	6	4
CA088.46DD	DD	4.6	3	II	CA.088	Woman	23	2	1	1	4	1	10

[1] Oral Health Index (OHI) values correspond to: 0= no plaque is observed; 1= plaque adheres to the probe; 2= plaque is observed in the gum region; 3= plaque is observed in 2/3 of the tooth.
[2] Gingival Index values correspond to: 0= healthy gingiva; 1= mild inflammation; 2= induced bleeding; 3= spontaneous bleeding. Brushing habits are indicated by the number of tooth brushes per day.
WS: white spot lesions; H: hidden dentin caries lesions; DD: deep dentin, open lesions. Caries lesion severity codes relate to: 1= non-cavitated enamel caries; 2= cavitated enamel lesions; 3= dentin-affecting lesions. Location codes refer to: I= occlusal areas and buccal or lingual pits; II= posterior interproximal; III= anterior interproximal; IV= anterior interproximal including incisal corner; V= gingival at facial or lingual.

3.3.2. RNA Extraction, PCR Amplification and Sequencing

RNA was extracted by a combination of physical and chemical lysis. Carious lesions samples were suspended in 500 ul sterile saline solution containing 0.1 and 0.5 mm glass beads, and subject to 50 Hz beating for 2 minutes in a Tissuelyzer II (QIAGEN) followed by 5 minutes on ice three times. Chemical lysis, RNA extraction and DNAse treatment was then performed using the RNA/DNA Masterpure Extraction kit (Epicentre) following the manufacturer instructions with the addition of a lysozyme treatment at 37 °C for 30 min.

Single-stranded cDNA was constructed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) in 20 ul reactions, with several modifications, following Benítez-Páez A et al. (2014). Universal bacterial primers 8F (5'-TCAGAGTTTGATCMTGGCTCAG-3') and 788R (5'-GGCCVGGGTATCTAATCC-3') were used to partly amplify the 16S rRNA gene from the single-stranded -cDNA in two 50 ul reactions, following the PCR and purification conditions described by Simón-Soro et al. (2013). In three cases, there was enough carious material to obtain both DNA and RNA. In those three individuals , DNA was also extracted from dental plaque and drooling saliva following Simón-Soro et al. (2013) and used for comparison. Purified PCR products were measured in a Modulus fluorimeter (Turner Biosystems) and mixed in equimolar amounts in two pools of 14 samples, which were sequenced in 1/8ths of a plate in the GS-FLX pyrosequencer (Roche) with Titanium-plus chemistry.

3.3.3. Sequence Analysis

To increase accuracy in taxonomic assignment, only reads longer than 400 bp were selected. Chimeric PCR products were filtered out using the software Uchime (Edgar RC et al., 2011) and the reads were end-trimmed and

quality filtered following Cabrera-Rubio R et al. (2013). Sequences were separated by the sample-specific 8-bp barcode and assigned to a genus using the Ribosomal Database Project classifier, with an 80% confidence threshold (Wang Q et al., 2007). Sequences were clustered at 97% nucleotide identity over 90% sequence alignment length and rarefaction curves were obtained using the RDP pyrosequencing pipeline. An attempt was done to assign the reads to the species taxonomic level. In order to do this, a curated database was constructed with the full-length 16S sequences of all species present in the Ribosomal Database Project that belonged to the genera *Streptococcus*, *Lactobacillus*, and *Veillonella*. A BlastN (Gertz EM et al., 2006) was then performed against this database with the reads that had been previously assigned to the above mentioned genera and that were >500 bp. The top hit from each sequence comparison was selected if the alignment length was >500 bp, and the sequence identity >99% (high stringency criterion).

3.4. RESULTS

After end-trimming and quality filtering, a total of 132,599 sequences were obtained for 15 enamel lesions and 12 dentin lesions. Chimeras reached 1.51% of the total and were filtered out. Averages of 4,911 reads were obtained per sample, with a mean length of 661bp.

Reads were clustered at 97% sequence identity, which is the consensus threshold for bacterial species boundaries (Yarza P et al., 2008). These Operational Taxonomic Units (OTUs) can therefore be used to estimate the number of species in a sample. Enamel caries lesions were the least diverse,

with a median of 177.7 bacterial species, whereas the estimates for open and hidden dentin cavities were 250.7 and 201.2, respectively (Figure 3.1).

Figure 3.1. Active bacterial diversity in caries lesions. Data show the estimated number of bacterial species (Operational Taxonomic Units, or OTUs), as calculated by the Chao Richness Index on the sequences clustered at 97% nucleotide identity. Patients' codes are displayed on the right panel and samples from the same individual are depicted with the same symbol. Data are shown for non-cavitated enamel caries (left), open dentin caries (middle) and unexposed, hidden caries (right). Medians from each group are shown for reference. Note that several caries samples are available for a few individuals.

Bacterial diversity levels varied not only between individuals but also between caries samples from the same individual, as also shown by rarefaction curves where the sequencing effort is plotted against the estimated number of species (Figure 3.2). The data suggest that white spot lesions appear to be a very restrictive niche, whereas open dentin cavities are the most diverse, even though only the inner layer of the lesion was selected for RNA extraction. Hidden dentin lesions were less diverse than deep dentin lesions from open dentin cavities, suggesting that the latter have a supply or microorganisms from the oral cavity. Open dentin lesions displayed also the highest variability, in accordance to their exposure to the salivary environment. The existence of such a high level of diversity even in the active fraction of the bacterial community, confirms that the high number of organisms detected in caries lesions is not due to dead or inactive species, and that dental caries is a polymicrobial disease, where multi-species microbial consortia are metabolically active in the lesions.

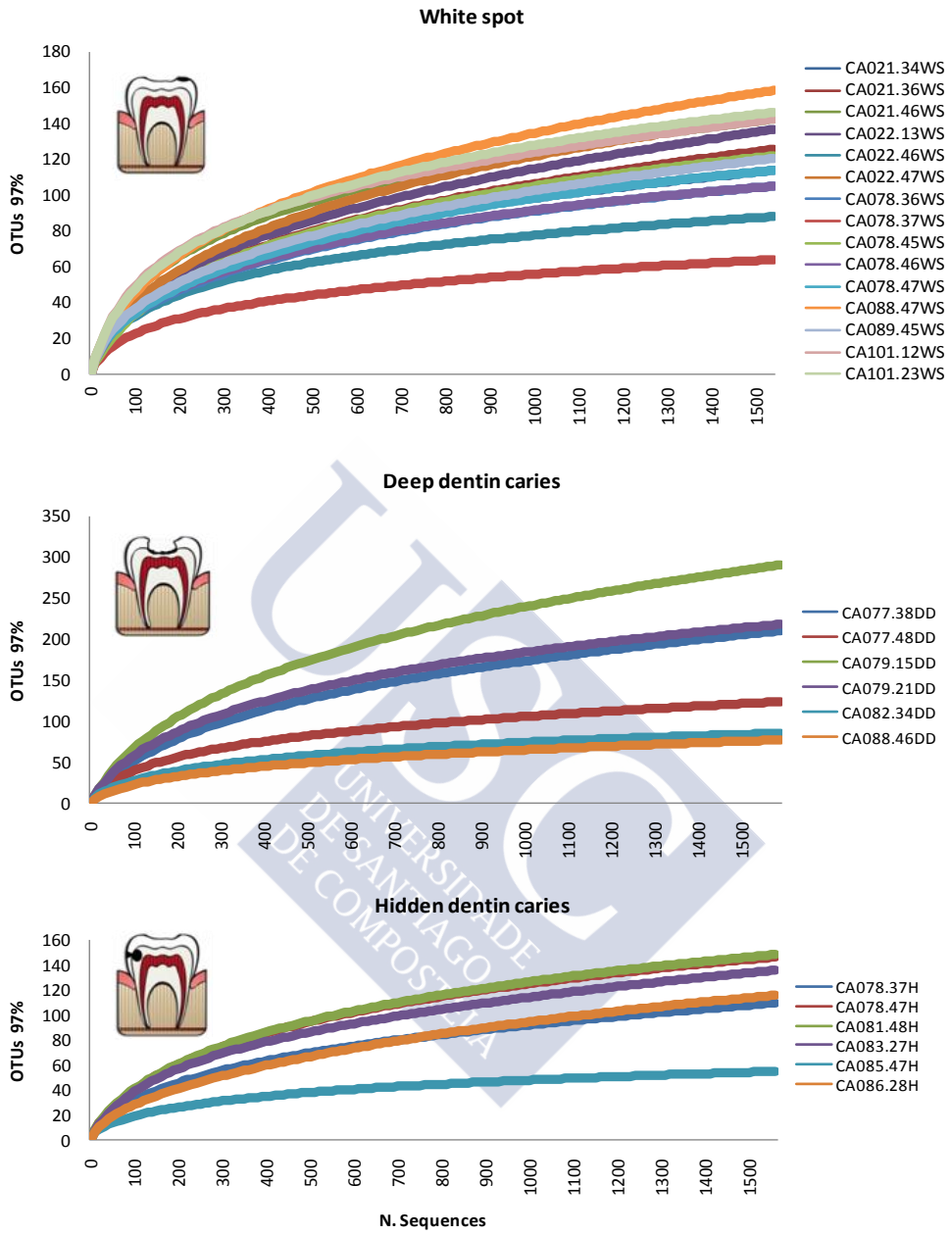

Figure 3.2. Rarefaction curves for individual carious samples. The curves relate the sequencing effort to the estimated number of species (Operational Taxonomic Units at 97% sequence identity). All curves were performed with the same number of sequences (randomly selected) so they are comparable. The numbers to the left of the dots indicate the patient's code, whereas the numbers to the right refer to the quadrant and tooth number. WS= white-spot lesion; DD= deep dentine lesion; H= hidden, closed dentine lesion.

Active bacterial composition was significantly different between enamel and dentin cavities (Unifrac distance p-value <0.001), suggesting that these microbial communities are tissue-dependent. Streptococci, *Rothia*, *Leptotrichia* and *Veillonella*, for instance, were at higher levels in enamel carious lesions, whereas *Lactobacillus*, *Shlegelella*, *Pseudoramibacter*, and *Atopobium* appeared to be clearly associated with dentin lesions (Figure 3.3, top). There is also a high number of minority species (found at <1% proportion, indicated as "Other" in Figure 3.3) that were exclusively found in enamel lesions and a few of them were only found in dentin carious lesions.

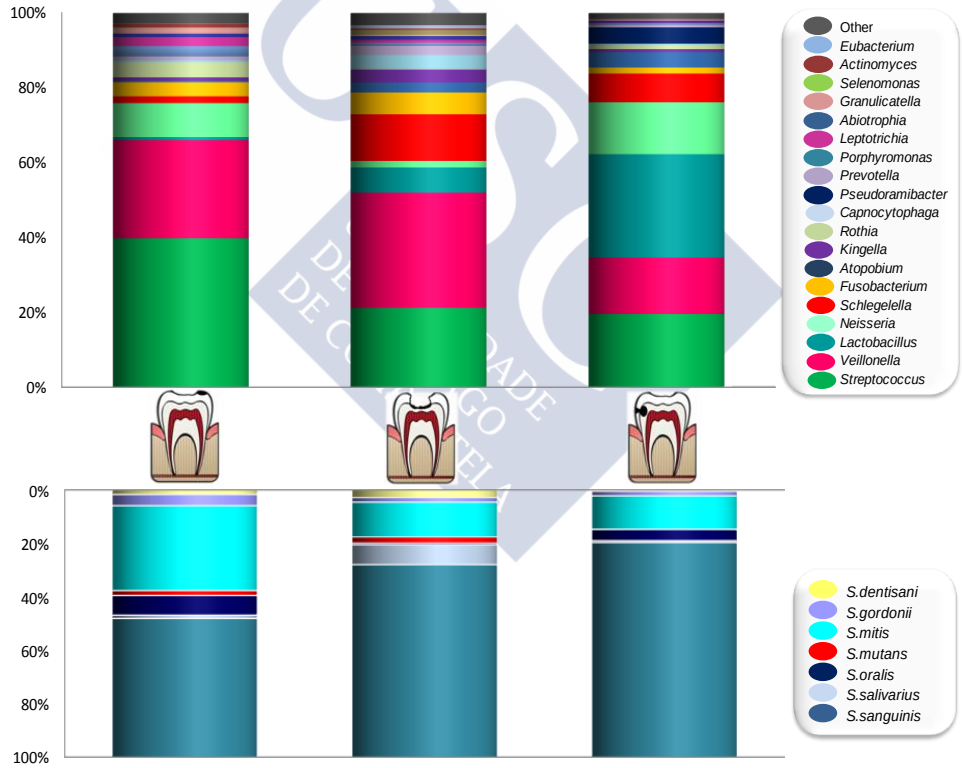

Figure 3.3. Taxonomic composition of active bacteria in caries samples as determined by pyrosequencing of the 16S rRNA gene. Graphs show the proportion of bacterial genera found at >1% of the total (top panel) and the proportion of different streptococcal species (lower panel), calculated as the means of all carious samples (n=15 for enamel lesions, n=6 for open dentin lesions, and n=6 for closed dentin lesions). Data are shown for non-cavitated enamel caries (left), open dentin caries (middle) and unexposed, hidden caries (right).

The latter included *Tannerella*, *Olsenella*, *Filifactor*, and *Treponema*. Given the high frequency of streptococci, an effort was made to identify streptococcal sequences at the species level (Figure 3.3, lower panel). *Streptococcus sanguinis* increased significantly in dentin cavities whereas *S. mitis* was more abundant in enamel lesions. In relation to *S. mutans*, which is probably the most studied caries-associated species, a dramatically low proportion was found in all samples, ranging from 0.73% in enamel lesions to 0.48% in open dentin and 0.02% in hidden dentin lesions. The low proportion detected confirms that this species is a minority (Gross EL et al., 2012; Simón-Soro A et al., 2013b) and questions its importance as the main etiological agent of tooth decay (Munson MA et al., 2004). Also, bacterial counts of lactobacilli frequently used to predict caries risk in diagnostic tests may not be informative given that these bacteria were found to be virtually absent in enamel lesions (Figure 3.4), and this would imply that they are probably not involved in caries initiation.

Bacterial composition in individual caries lesions showed highly variable microbial consortia both between and within individuals. There was only one case in which the lesion was dominated by a single bacterial genus (marked as sample CA085, tooth 47 in the Figure 3.4). This exceptional instance involved *Lactobacillus*, which was found to represent 99% of the RNA-based population in a hidden dentin cavity (55% of the reads had maximal similarity to *Lactobacillus gasseri*, but three other species of lactobacilli were present).

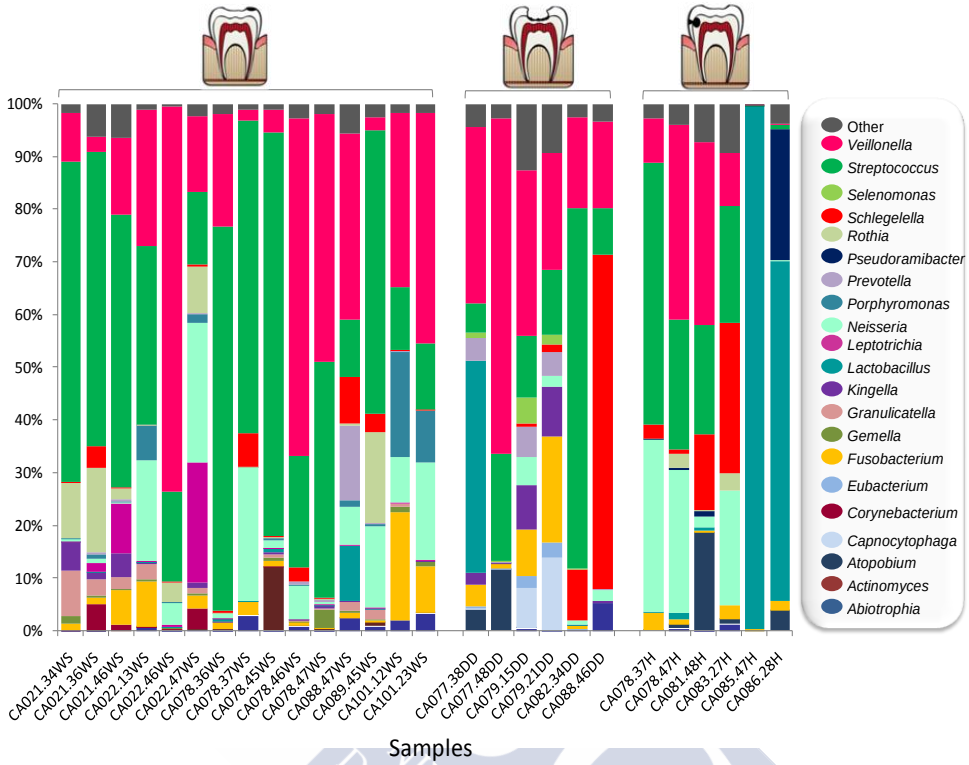

Figure 3.4. Metatranscriptomic profile of individual caries lesions. RNA was extracted from initial, white-spot enamel caries (WS), the deep layer from open dentin cavities (DD), and hidden dentin cavities (H). After cDNA construction, bacterial taxonomy was obtained by Titanium-plus pyrosequencing of 800 bp PCR products encompassing hypervariable regions V1 through V4 and using the Ribosomal Database Project (RDP) classifier. Over 4,000 reads per sample were obtained, giving information about the active microbial community. The two numbers to the right of the patient code indicate the tooth from where the sample was obtained.

There were three cases in which DNA from the same caries lesions, together with saliva and supragingival dental plaque DNA from sound teeth surfaces could also be obtained. These pilot data show that when the DNA-based bacterial composition of a lesion is compared to the RNA-based composition of the same individual lesion, a clear difference was observed

(Figure 3.5, inner circles). The former, metagenomic approach, shows the total bacterial community whereas the metatranscriptomic approach describes the active players in that community and we propose the latter procedure is therefore a closer approximation to the disease etiology. Other samples taken from the oral cavity of the same individual, like supragingival dental plaque or unstimulated saliva, show a different bacterial composition (Figure 3.5, outer circles), where the detection of cariogenic bacteria is hampered by the predominance of microorganisms from other buccal niches shown in Chapter 1. For instance, *Megasphaera* is found in saliva but passed virtually undetected in caries lesions, and *Porphyromonas* was found at considerable proportions in saliva and supragingival dental plaque, but is a minority in enamel lesions.

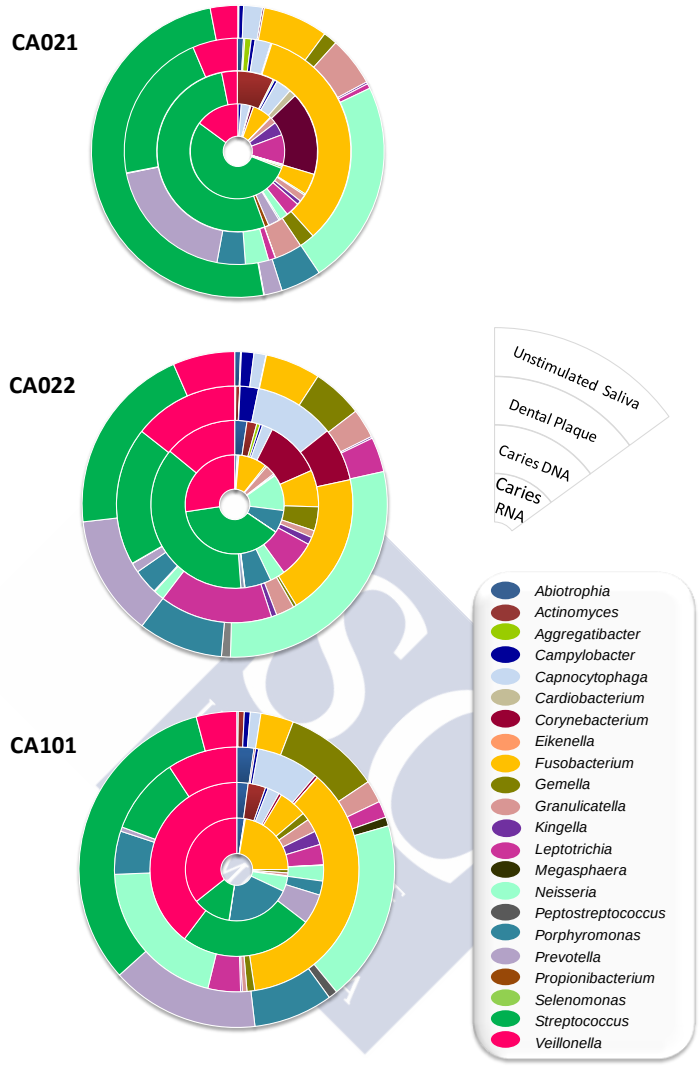

Figure 3.5. Bacterial composition in different oral samples from three caries bearing individuals. Data show the proportion of bacterial genera for unstimulated saliva (DNA-based), supragingival dental plaque of sound teeth surfaces (DNA-based), enamel caries lesions (DNA-based) and enamel caries lesions (RNA-based), from the outer to the inner circles.

3.5. DISCUSSION

The number of active species in lesions found in the current work is lower than in previous studies based on ribosomal DNA, from 206 in white spot lesions and 379 in dentin lesions shown in Chapter 2 to 177 and 201 found in this study, respectively. This list can be taken as a first approximation of the repertoire of microorganisms potentially involved in caries initiation and progression, as the taxonomic description of caries lesions is a vital step in determining the etiology of the disease. Several taxonomic groups are nevertheless likely to be under-represented, such as G+C rich bacteria including *Actinomyces* and *Bifidobacterium*, which are known to be poorly amplified by "universal" primers (Cabrera-Rubio R et al., 2012). Mutans streptococci, however, are readily amplified with the primers used in this and other studies (Munson MA et al., 2004), and their low proportion is probably not a PCR artifact, as also proposed by Chapter 2 of present Thesis and other authors in DNA-based studies (Gross EL et al., 2012).

It has also to be borne in mind that fungal species have also been detected in caries lesions, and proposed to contribute to cariogenicity (Falsetta ML et al., 2014). Thus, the use of bacterial primers cannot detect fungal organisms and it would be necessary to perform high-throughput sequencing analyses of fungal species to gain insights into the diversity and contribution of these microorganisms to dental caries. Nevertheless, the varying polymicrobial nature of cavities shown in this Chapter and the currently accepted ecology-based hypothesis of caries disease (Marsh PD, 2003; Takahashi N and Nyvad B, 2011) underline that the functional output of the microbial community is probably more important than its species-composition in order to understand and combat the disease. Thus, in the future, the application of high-throughput direct sequencing to the RNA extracted from oral samples (Benítez-Páez A et al.,

2014; Duran-Pinedo et al., 2014) will provide an opportunity to identify not only the active microbial composition but also the expressed genetic repertoire underlying disease initiation and progression. From an applied point of view, the polymicrobial etiology of dental caries underlines that diagnostic and therapeutic strategies directed at single species (e.g. passive and active immunization) are likely to be unsuccessful.

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4

Revealing Microbial Recognition by Specific Salivary Antibodies

Simón-Soro A, D'Auria G, Collado MC, Dzunkova M, Culshaw S, Mira A. *Revealing microbial recognition by specific antibodies*. BMC Microbiol. (2nd revision)

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4.1. ABSTRACT

Recognition of microorganisms by antibodies is a vital component of the human immune response. However, there is currently very limited understanding of immune recognition of 50% of the human oral microbiota which is made up of as yet un-cultured bacteria. In addition, given that enamel is the only oral tissue which is inert, it does not have a mucosal immune system, and there is little knowledge available about the contribution of salivary antibodies in preventing dental caries. Ihave combined the use of flow cytometry and pyrosequencing to describe the microbial composition of human oral samples and its interaction with the immune system.

I have set up the technique in saliva and dental plaque samples by using fluorescent markers which are specific for IgA, IgG or IgM. By the use of Fluorescence-Activated Cell Sorting (FACS), bacterial cells can be separated depending on whether they are coated with specific human antibodies. Each bacterial population can then be PCR-amplified and pyrosequenced, characterizing the microorganisms which evade the immune system and those which are recognized by each immunoglobulin.

The application of the technique to caries-free and caries-active individuals (n=10 on each group) shows that healthy individuals have significantly higher levels of Ig-coating in saliva, and that the taxonomic composition of Ig-coated bacteria is different from that found in diseased individuals. This suggests that the immune response in caries-free individuals is more competent and plays a role in preventing dental caries.

4.2. INTRODUCTION

Large efforts have been developed to describe the human microbiota and germ free animal models demonstrate its intricate relationship with the host (Hooper et al., 2012). However, less is understood about this relationship in humans, under health and disease conditions. The coating of microorganisms by antibodies may promote defense against infection and play a role in regulating the immune response to the microbiota to limit potentially damaging responses, thus maintaining homeostasis in different human-associated microbial communities (Hooper et al., 2012; Mshvildadze and Neu, 2010). Recognition of microbes by different immunoglobulins (Igs) play a vital role in the host-microbiome relationship (Brandtzaeg, 2010), but the precise associations between each Ig type and specific groups of bacteria and fungi are poorly characterized by current techniques (Woof and Mesteck, 2005). In the oral cavity, the role of Ig-coating in preventing dental caries has been poorly studied(Marcotte H and Lavoie MC, 1998), and is limited to several cariogenic species like *Streptococcus mutans* (Malcolm et al., 2014). However, advances in flow cytometry now allow the separation of bacterial cells according to their population structure (Koch et al., 2013; Peris-Bondia et al., 2011) and to the fluorescence emitted by secondary antibodies that specifically bind to different human Igs (Tsuruta et al., 2010; Tsuruta et al., 2012). In addition, second-generation sequencing of PCR-amplified microbial rDNA genes provides a description of the bacterial and fungal diversity and taxonomic composition in small human samples (Human Microbiome Project Consortium, 2012).

We have applied a combination of flow cytometry cell sorting coupled with pyrosequencing to, without the culturing bias, identify the bacteria that are coated with specific antibodies. In the first part of this Chapter, I have set up this mixed approach in saliva and oral biofilm (supragingival dental plaque) samples, by designing and optimizing a protocol that allows estimating the proportion of

Ig-coated bacteria as well as identifying the specific bacterial genera coated and uncoated by different Igs. In the second part of this Chapter,I have applied this methodology to saliva samples from caries-free and caries-bearing individuals, in an attempt to determine the potential role of bacterial recognition by salivary antibodies in preventing or facilitating dental caries.

4.3. METHODS

4.3.1. Sample Collection and Processing

For studying the potential role of Ig-recognition on dental caries, an intraoral examination was performed on 20 selected subjects, gathering information on: presence of caries, plaque deposits (Oral Hygiene Index, OHI) (Green and Vermillion, 1960), presence of gingival bleeding (Löe and Silness gingival index, GI) (Löe and Silness, 1963), following recommendations and nomenclature from the World Health Organization (WHO, 1997). They had not been treated with antibiotics in the three months prior to the study nor presented antecedents of routine use of oral antiseptics. They were all adults, aged 19-39 years, and had no missing teeth. Ten subjects had no history of dental caries (DMF index = 0) and the other ten had active cavities at the moment of sampling, with or without a history of dental caries (fillings).All donors signed an informed consent and the sampling protocol was approved by the CSISP-DGSP ethical committee (Valencian Health Authority, Spain).

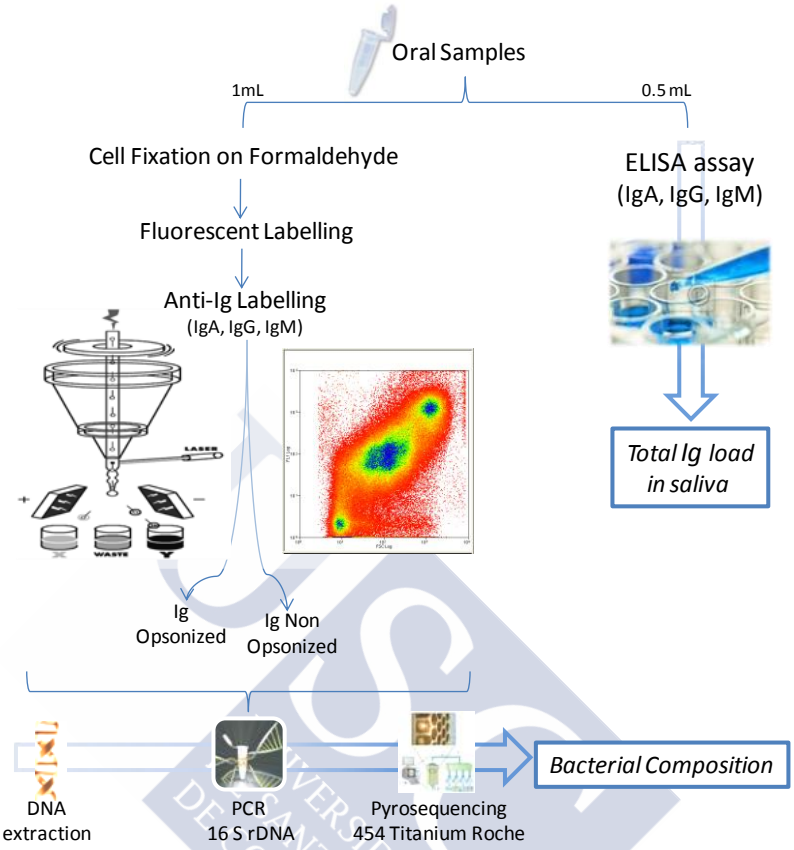

Figure 4.1. A mixed Flow Cytometry-Next Generation Sequencing strategy to identify human host-microbial associations. Saliva and oral biofilm samples are disaggregated by vortexing and mild sonication. Microbial cells are fixed in 4% paraformaldehyde previously to staining with fluorescent markers to detect cells and specific antibodies (anti-human Ig) through a flow cytometer. Microbial load can also be accurately estimated by cell counting. Cells are sorted depending on whether they are opsonized with either IgA, IgG or IgM antibodies, o by all three simultaneously. Each bacterial population can then be PCR-amplified and pyrosequenced, characterizing the microorganisms which evade the immune system and those which are recognized by each immunoglobulin. The application of the technique to healthy and diseased individuals may unravel the contribution of the immune response to microbial infections and oral polymicrobial diseases.

All donors brushed their teeth at 9-10 am using water, to prevent any potential effect of toothpaste on salivary composition and characteristics. Non-estimulated saliva samples were taken by drooling 30 min (0h), 6 h, 12 h and 24 h after toothbrushing, collecting it in a sterile 50 mL Falcon tube avoiding

spitting or plaque removal by the tongue (Navazeshand Christensen, 1982), up to a total of 5 mL.

For setting up the protocol(Figure 4.1), drooling saliva and dental plaque samples were collected as in Chapter 1 from 12 volunteers, aged 19-39 years, 24 hours after toothbrushing using water.

Half of the volume from all samples was centrifuged for 10 minutes at 6,000 x g. The pellet was washed once in sterile saline solution, fixed overnight in 4% paraformaldehyde and stored in 50% ethanol at -20 °C until used for flow cytometry. The other half of the volume was stored at -80 °C until used for ELISA tests, or PCR.

The observation of unstimulated saliva samples by fluorescence confocal microscopy, and electron microscopy showed that bacteria were normally forming aggregates, either with other bacteria or most frequently with human epithelial cells (Figures 4.2 and 4.3).Thus, on the experiment day, samples were washed in sterile saline solution and disaggregated 20 seconds in a Raypa VCI-50 sonicator at low ultrasound intensity. This was found to be sufficient to disaggregate bacteria, as corroborated by light and confocal microscopy, but still keeping cell integrity, as seen by the absence of cell debris in flow cytometry scatterplots.

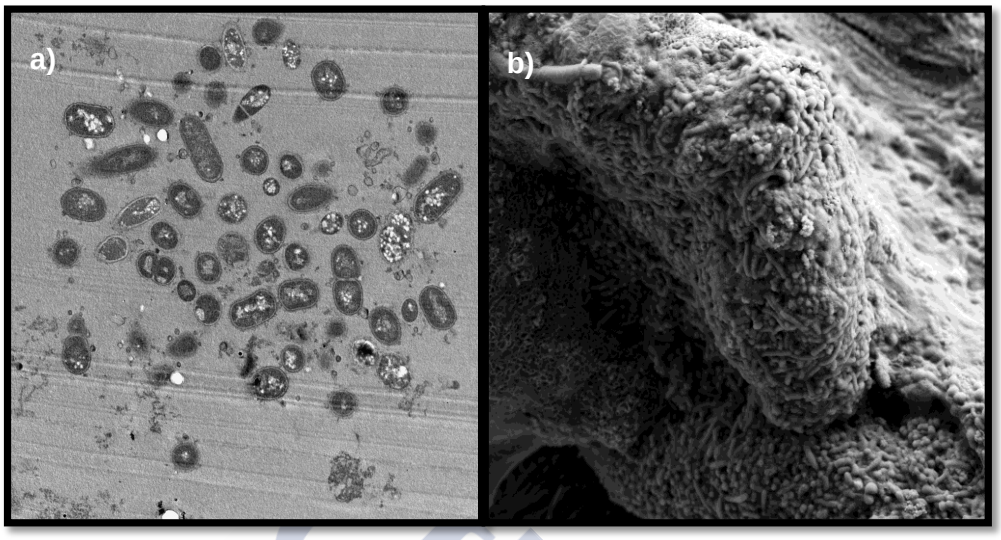

Figure 4.2.Bacterial aggregates in unstimulated human saliva. (a) Transmission electron microscopy image showing a cluster of microorganisms (magnification: 24.000X). (b) Scanning electron microscopy photograph showing an oral epithelial cell partly coated with a dense microbial aggregate (magnification: 5.960x).

4.3.2. Flow CytometryProtocol

Samples were suspended in 5% albumin to prevent non-specific antibody binding, then stained with (i) anti-human Ig labelled with FITC (Invitrogen); and (ii) the DNA-binding fluorophore SYTO62 (Invitrogen) according to the manufacturer's instructions. Anti-mouse Ig labelled with FITC (Invitrogen) was used for isotype control (Figure 4.1).

Cell sorting was performed with the MoFloTM XDP flow cytometer using Argon 488 nm (blue) laser (200 mW power) and the 635 nm (red) diode laser (25 mW power) as light sources. The lasers were aligned using Flow-CheckTM (10µm) and Flow-SetTM (3µm). Emission filters were 520/30 for FITC and 680/30 for SYTO62 respectively. Proper fluorescent marking was assessed by fluorescence and confocal microscopy (Figure 4.3). Cells were separated

according to their fluorescence in both the FITC and SYTO62 channels (Ig-coated bacteria) and the SYTO62 channel only (non-coated bacteria).

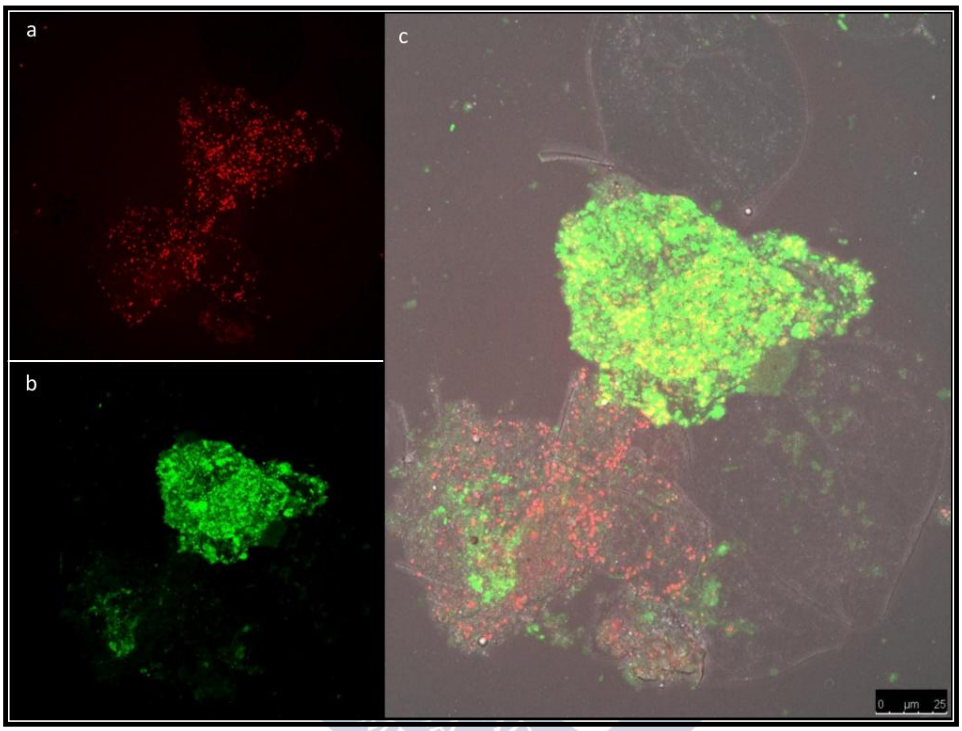

Figure 4.3. Staining of microbial cells for Fluorescence Activated Cell Sorting. Confocal laser microscopy images of intact, non-sonicatedsaliva samples collected 30 min after toothbrushing from individual NCATV01 stained with different fluorescent markers (using a 63x magnification and a 1.5 zoom on a Leica TCS SP2 laser scanning spectral confocal microscope with an HCX APOL 63x/0.9 water-immersion lens). (a) Transversal section corresponding to the saliva sample stained with the fluorophore SYTO62 (DNA labeling, red fluorescence channel). (b) Transversal section corresponding to the saliva stained with fluorophore FITC (anti-human IgG, green fluorescence channel). (c) A maximum projection of all transversal sections in the sample(superposition of all planes captured with bacterial DNA and Ig-binding markers).Both stained and unstained bacteria are observed, as well as human epithelial cellsto which they are attached, thanks to trans-illumination. Bacterial cells can now be separated, after sonication, by fluorescence detection cell sorting in order to study the corresponding Ig-coated and uncoated microbial populations.

4.3.3. Taxonomic Identification

DNA from the Ig-coated and non-coated fractions with more than 5,000 cells was extracted by a combination of physical and chemical lysis, and the 16S

rRNA gene amplified using universal bacterial primers 8F and 533R with sample-specific barcodes, as previously described (Chapter 1, Simón-Soro et al., 2013b). Purified PCR products were mixed in equimolar amounts and sequenced using the 454 GS-FLX pyrosequencer (Titanium chemistry, Roche). Obtained 16S rRNA reads were end-trimmed in 10pb sliding windows, then length (200 bp) and quality filtered (average Q>20). Taxonomic assignments were performed with the RDP classifier (Wang et al., 2007), and to estimate total diversity, sequences were clustered at 97% nucleotide identity over 90% sequence alignment length to obtain rarefaction curves, which were normalized by same number of sequences per group. Principal Coordinates Analysis (PCoA) was performed with FastUnifrac (Lozupone, 2006), comparing the 16S-estimated composition with a phylogenetic approach that takes into account both taxonomically assigned and unassigned reads.

4.3.4. Measurement of Ig Levels

Enzyme-linked immunosorbent assays (ELISA) were performed by duplicate to measure the salivary concentrations of immunoglobulins, using the Human IgA, IgG and IgM ELISA kits (Hycult Biotech) following the manufacturer's recommendations, with sample dilutions1:1,000, 1:10 and 1 for IgA, IgG and IgM, respectively. Absorbance was measured at 450 nm with a TecanInfinite F200 PRO plate reader at room temperature.

4.3.5. Measurement of Bacterial Load

Bacterial cell densities were calculated by quantifying the number of bacterial cells per mL of saliva by qPCR of the extracted DNA, using universal primers designed to amplify the single-copy gene fusA (Santos SR and Ochman H, 2004). The amplification was done with an annealing temperature of 62°C in a LightCycler 480 Real-Time PCR System (Roche). Each reaction mixture of 10 µl

was composed of SYBR Green PCR Master Mix (Roche), 0.5 µl of the specific primer (10 µM) and 2 µl of DNA template. All amplifications were performed in duplicates and the bacterial concentrations were calculated using obtained Ct-values with standard curves obtained by relating Ct-values to a known number of bacterial cells as determined by flow cytometry (Boix A, 2014).

4.3.6. Statistical Analysis

Due to the small sample size, non-parametric tests were applied to analyze the different study variables. For the comparisons between Ig-coating levels in saliva and biofilm (set up experiments), a Wilcoxon test was used. For comparing Ig-coating levels, Ig levels and bacterial load between caries-free and caries-active individuals, the Mann-Whitney U-test was used. For comparing Ig-coating levels, Ig levels and bacterial load at different times within the same group of individuals, the Friedman test and the Wilcoxon signed-rank test (for pairwise comparisons) were used. Correlations between Ig-coating levels and salivary Ig concentration,and between bacterial load and Ig-coating levels were analyzed by using the Spearman's Rho coefficient, whereas the correlation betweenIgconcentrations and bacterial load were identified through linear regression estimates. Significance was considered at p-values <0.05. Data were analyzed with PASW Statistics Base 20 package for Windows (IBM).

4.4. RESULTS

PART A. Set-up of the Protocol

Formaldehyde-fixed samples were stained with (i) anti-human IgA, IgG or IgM labelled with FITC; and (ii) the DNA-binding fluorophore SYTO62. Proper fluorescent marking was assessed by fluorescence and confocal laser scanning microscopy (Figure 4.2). Anti-mouse IgA, IgM or IgG labelled with FITC (Invitrogen) were used to control for non-specific binding (isotype control). Cells were separated in the flow cytometer according to their fluorescence in both the FL1-FITC and FL8-SYTO62 channels (Ig-coated bacteria) and the SYTO62 channel only (non-coated bacteria). An improvement in preventing non-specific binding was observed by incubation with albumin. DNA from the Ig-coated and non-coated fractions was extracted and the 16S rDNA gene amplified and pyrosequenced, in order to describe bacterial composition of the sorted populations. The density and number of cells that could be sorted from each fraction varied among samples (Table 4.1). Although PCR and subsequent pyrosequencing was achieved with as few as 5,000 sorted bacteria, a larger number of cells are recommended for accurate description of microbial composition in samples with small cell counts (Biesbroek et al., 2012).

All samples were taken 24 hours after toothbrushing, so the dental plaque in these individuals can be assumed to have a formation time of 24 h. The average proportion of IgA-opsonized bacteria in the subjects analyzed in this proof of concept study ranged from 73.6% for saliva to 78.4% for the oral biofilm (Table 4.1), although some caries-bearing individuals had extremely low levels of Ig-coating. There was greater fluorescence intensity in IgG-coated bacterial populations compared with IgA in saliva (an average of 93.8% of bacteria were IgG-coated) and oral biofilm samples (92.1% of bacteria were IgG-coated). This could potentially reflect a biological difference in the corresponding antibodies

specificity in oral samples but different efficiencies in anti-human Ig markers binding or in fluorescence emission cannot be ruled out.

Table 4.1. Estimated number of Ig-coated cells as detected by Fluorescence-Activated Cell Sorting in salivaand oral biofilm samplescollected 24 hours after toothbrushing.

	Ig type	Total number of events (*)	Events not stained	Events stained	Estimated % opsonization (1)	Estimated % opsonization (2)
		Saliva Samples				
NCATV.01	IgA	277519	24000	24827	50.85	91.35
NCATV.01	IgG	317523	313	292643	99.89	99.90
NCATV.03	IgA	2329	907	902	49.86	61.06
NCATV.03	IgG	3379	1189	1501	55.80	64.81
NCATV.13	IgA	64277	331	21999	98.52	99.49
NCATV.13	IgG	52681	90	23514	99.62	99.83
NCATV.12	IgA	52489	794	9689	92.43	98.49
NCATV.12	IgG	39581	300	8024	96.40	99.24
NCATV14	IgA	7818	79	5939	98.69	98.99
NCATV.14	IgG	5736	19	5042	99.62	99.67
NCATV.15	IgA	35682	2826	7651	73.03	92.08
NCATV.15	IgG	8144	8	7012	99.89	99.90
NCATV.16	IgA	46420	391	34086	98.87	99.16
NCATV.16	IgG	56675	48	55120	99.91	99.92
CATV.54	IgA	230726	199300	1407	0.70	13.62
CATV.54	IgG	121442	19563	13018	39.96	83.89
CATV.57	IgA	13154	3523	351	9.06	73.22
CATV.57	IgG	7648	605	3649	85.78	92.09
CATV.58	IgA	4201	891	1686	65.42	78.79
CATV.58	IgG	3812	134	3011	95.74	96.48
CATV.59	IgA	713765	367303	12357	3.25	48.54
CATV.59	IgG	29276	966	24839	96.26	96.70
CATV.60	IgA	1497908	858524	26979	3.05	42.69

	Ig type	Total number of events (*)	Events not stained	Events stained	Estimated % opsonization (1)	Estimated % opsonization (2)
		Oral Biofilm (dental plaque)				
NCATV.01	IgA	18184	2372	3711	61.01	86.96
NCATV.01	IgG	22496	411	14326	97.21	98.17
NCATV.03	IgA	92386	17260	19580	53.15	81.32
NCATV.03	IgG	21918	1213	11427	90.40	94.47
NCATV.13	IgA	6341	720	1880	72.31	88.65
NCATV.13	IgG	18137	121	8910	98.66	99.33
NCATV.12	IgA	68915	2946	13444	82.03	95.73
NCATV.12	IgG	54898	390	22898	98.33	99.29
NCATV14	IgA	651156	7032	220752	96.91	98.92
NCATV.14	IgG	617821	276	383777	99.93	99.96
NCATV.15	IgA	755467	1056	355902	99.70	99.86
NCATV.15	IgG	1061756	3168	445649	99.29	99.70
NCATV.16	IgA	250136	89	200327	99.96	99.96
CATV.54	IgA	21936	12466	284	2.23	43.17
CATV.54	IgG	17576	1605	4383	73.20	90.87
CATV.57	IgA	7412	4928	34	0.69	33.51
CATV.57	IgG	2885	854	126	12.86	70.40
CATV.58	IgA	2387	617	402	39.45	74.15
CATV.58	IgG	4254	163	3179	95.12	96.17
CATV.59	IgA	125125	15812	44696	73.87	87.36
CATV.59	IgG	136475	6532	54707	89.33	95.21
CATV.60	IgA	119635	44510	26248	37.10	62.80
CATV.60	IgG	96302	22438	25723	53.41	76.70
CATV.61	IgA	42324	15679	5141	24.69	62.95
CATV.61	IgG	93251	9776	37207	79.19	89.52

(*) indicates the number of events marked by the DNA-labelling fluorophore SYTO62; its number is larger than the addition of the following two columns, as it includes the events marked with anti-mouse Ig (potential non-specific binding).
(1) Estimated Ig-coated cells calculated as a proportion out of the addition of opsonized and non-opsonized sorted events.
(2) Upper estimate of opsonization, calculated as a proportion of total events, including potential non-specific binding (1-non-stained/total cells).

These levels of Ig-coating are larger than those observed in the gut: Forty-five percent of faecal bacteria were found to be opsonized by IgA under health conditions, a proportion that increased to 69% under inflammatory episodes (van der Waaijet al., 2004).However, given that in the gut, the number of secreted IgA was estimated to be 10^7 times higher than the number of microbes (Conley and Delacroix, 1987), higher proportions can potentially be achieved. The greater proportion of opsonized bacteria detected in the oral samples analyzed in the current study is intriguing and could reflect local immune regulation requirements or an antibody mediated defense to limit microorganisms binding the gastrointestinal tract. It is also interesting to note that dental plaque samples did not present lower Ig-coating levels. Thus, even ifIg-free bacteria preferentially form the oral biofilm, Ig-coating must still be possible on the biofilm itself, at least on the outer layers. There is also the possibility that the sonication protocol did not suffice to break the aggregated cell structure of dental plaque, distorting opsonization level measurements.

IgA- and IgG-coating levels were significantly larger in caries-free individuals compared to diseased individuals, both in saliva and biofilm samples (Figure 4.4). This indicates that the immune response activity in caries-free individuals could be more competent than in patients with compromised oral health, as later confirmed in the second study, when the total Ig-coating levels of bacteria by IgA, IgG and IgM were estimated.

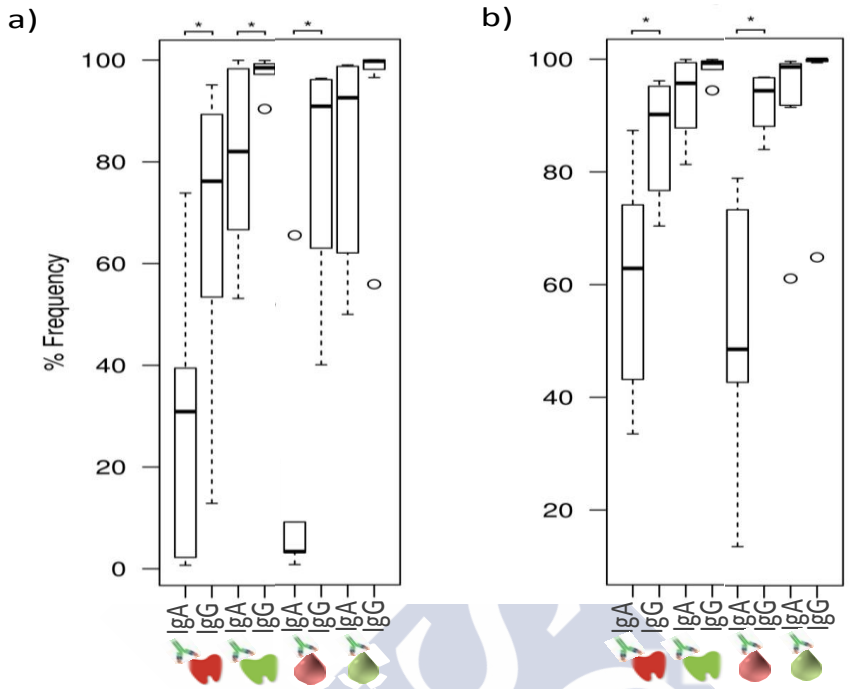

Figure 4.4. Proportion of Ig-coated and uncoated bacteria in oral samples collected 24h after toothbrushing. Boxplots show the mean values and variation in IgA- and IgG-opsonization levels for oral biofilm (tooth symbol) and saliva (drop symbol). Asterisks indicate statistically significant differences between IgA and IgG coating (non-parametric Wilcoxon test, $p<0.05$). For saliva and oral biofilm, samples from caries-free (green icons) and caries-bearing (red icons) individuals are available. Data are shown for a conservative (a) and a non-conservative (b), upper estimate of Ig-coating.

Pyrosequencing of Ig-marked and unmarked cells identified bacteriawhich were opsonized and non-opsonized by specific antibodies. In both saliva and oral biofilm samples, the frequency of many bacterial genera in the two sorted fractions was different, suggesting a particular affinity of the antibody for some microorganisms (Figure 4.5). As a consequence, specific bacteria appeared to be able to evade opsonization in some individuals, like the caries-associated bacteria *Abiotrophia* and *Lactobacillus* in saliva samples. Others, like *Veillonella* and *Fusobacterium*, appear always opsonized both in saliva and

dental plaque. In dental plaque samples, most bacterial genera appeared to be IgA-coated.

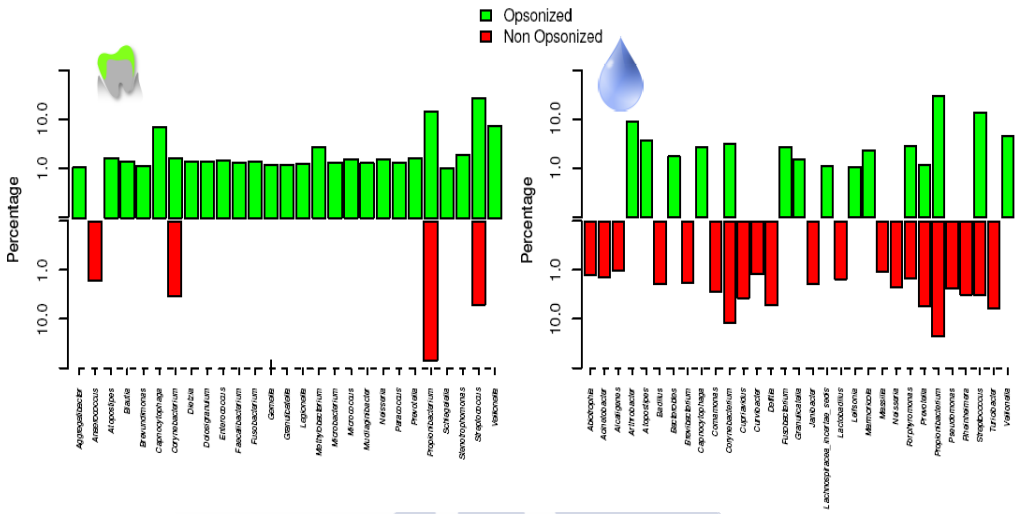

Figure 4.5. Bacterial composition of IgA-coated and non-coated populations in a caries-bearing individual. Graphs show the proportion of bacterial genera within the opsonized and non-opsonized populations in individual samples from oral biofilm (dental plaque) and saliva collected 24h after toothbrushing, as estimated by 16S rDNApyrosequencing of fluorescence-activated sorted cells. Only bacteria found at a frequency >1% are shown. Some bacterial genera appear at similar proportions in both the Ig-coated and non-coated populations. Others appear only within the opsonized fraction (strong IgA-specificity) whereas some microorganisms are present only within the non-opsonized fraction (immune evasion or non-recognition).

The sum of the proportion of IgA- and IgG-coated bacteria for the same samples was in most cases higher than 100%, indicating that a large proportion of cells were in fact coated by both immunoglobulins, as suggested by other studies (Green 1993, d'Auriaet al., 2013). However, the bacterial composition in the IgA- and IgG-coated fractions was different (Figure 4.6).

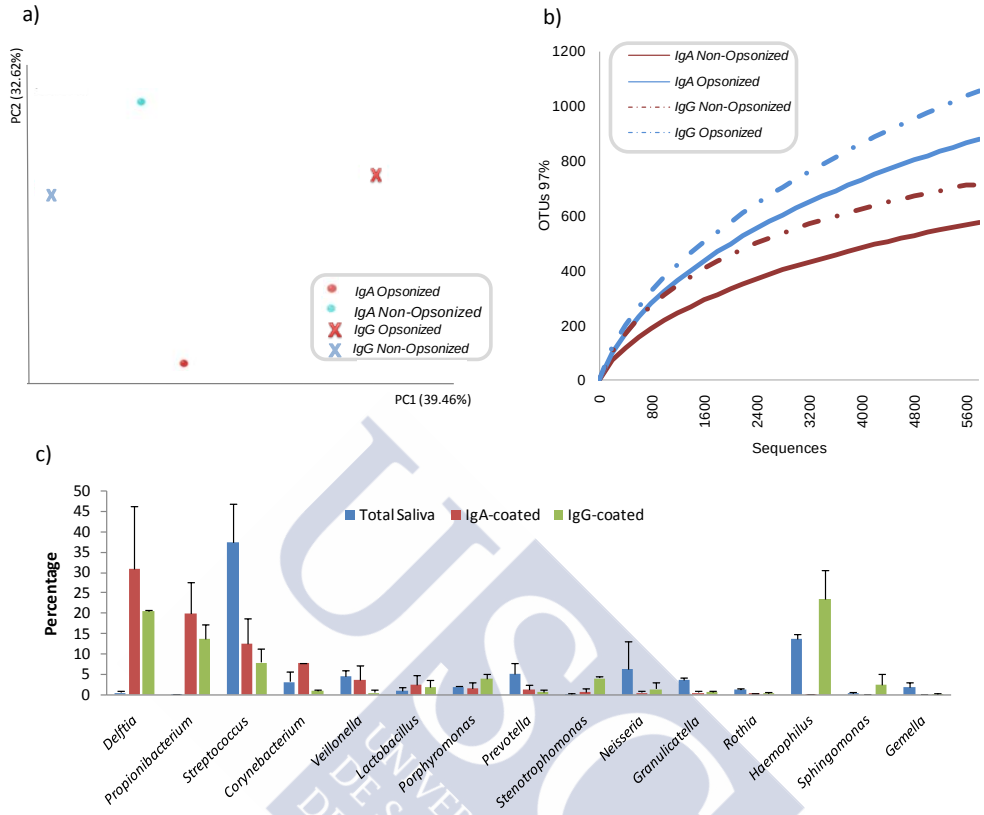

Figure 4.6. Diversity of Ig-coated and uncoated bacteria in human saliva. Saliva samples collected 24 h after toothbrushing (n=16) were stained with fluorescent markers for bacterial DNA, IgA and IgG, and sorted in three groups: IgA-coated bacteria, IgG-coated bacteria and uncoated, non-opsonized bacteria. (a)Principal Coordinates Analysis of samples, performed according to bacterial composition, as estimated by pyrosequencing of the 16S rDNA gene. (b) Rarefaction curves relating pyrosequencing effort to the estimated number of species (OTUs at 97% sequence identity). (c) Bacterial composition at the genus level for total saliva samples (prior to cell sorting), as well as for the IgA-coated and IgG-coated fractions. The bacterial composition appeared to be different also in the IgA-coated and IgG-coated fractions. The non-opsonized fractions display a lower diversity and different taxonomic composition from opsonized populations.

As shown by a Principal Coordinates Analysis (PCoA)in saliva samples, the composition of the IgA- and IgG-opsonized fractions did not cluster together (Figure 4.6). In addition, the opsonized and non-opsonized populations occupied different positions in the PCoA space, indicating that the taxonomic groups coated by antibodies are different from those that are ignored or undetected by

them. For instance, data for saliva samples show that *Propionibacterium* or *Delftia*, which was found at low levels in the sample, appeared to be highly opsonized by both IgA and IgG, whereas *Streptococcus* and *Neisseria*, which were found at high proportions in the saliva sample, occupied a much smaller fraction in the IgA-and IgG-coated populations (Figure 4.6c). In the future, the massive sequencing of IgA-, IgG- and IgM-coated microbes in larger sample sizes should confirm whether there is Ig-specific opsonization.

Finally, rarefaction curves of species-level bacterial diversity show that the opsonized population is more diverse than the non-opsonized one (Figure 4.6b). In future studies, we anticipate that the sequencing of the non-opsonized fractions will reveal those microorganisms that are undetected or ignored by specific antibodies. Although the current work was done with titanium chemistry FLX pyrosequencing and sequences were under 500 bp long on average, current advances in this technology and in third-generation sequencing are expected to allow read lengths over 1000 bp shortly, allowing taxonomic assignment at the species level. This will no doubt be necessary for accurate description of antibody-microbial specificity, as current read lengths are mainly reliable at the genus level (Cole et al., 2009).

A feature that can readily be observed in flow cytometry scatter plots in environmental samples is the presence of aggregated populations as evidenced by their larger size and specific shapes (Koch et al., 2013). Our own observations in human oral samples through fluorescence and confocal microscopy revealed that some of those large-size clusters are bacterial aggregates and others are formed by bacteria bound to host cells like detached buccal epithelial cells (Figures 4.2 and 4.3). These aggregates can easily by sortedwhen intact, unsonicated saliva samples are used and subsequently identified by 16S rDNApyrosequencing (Figure 4.7). In individual CA060, for instance, 70% of a bacterial aggregate in a saliva sample was found to be

formed by *Porphyromonas*, *Streptococcus*, *Prevotella, Propionibacterium*, *Veillonella*, and unidentified *Bacteroidetes*. This approach paves the way to unravel the nature of bacterial aggregation in body fluids with important repercussion for active and passive immunization strategies. For instance, aggregated microorganisms may be less accessible to antibodies and partially escape opsonization in anti-caries vaccination approaches.

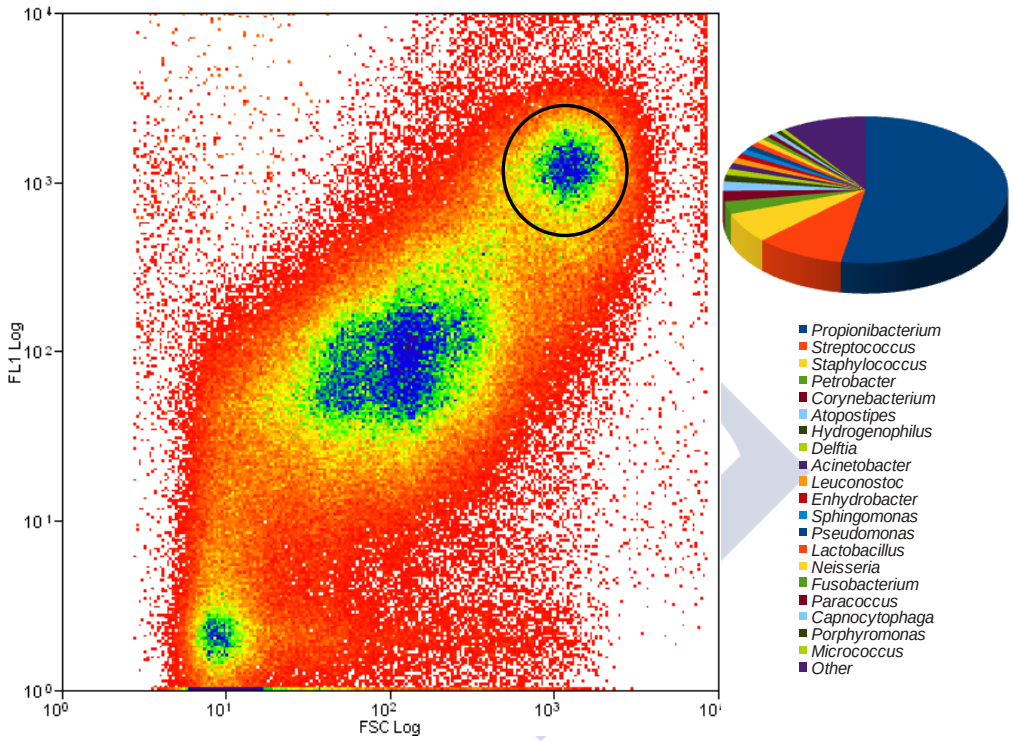

Figure 4.7. Identification of bacterial composition in aggregates. The scatterplot shows microbial cells in a saliva sample according to their size (X axis) and the IgG coating, as indicated by their FITC-fluorescence using anti-human IgG markers (Y axis). The large-size aggregate (indicated with a black circle) was separated by fluorescence-activated cell-sorting and its DNA pyrosequenced after PCR of the 16S rRNA gene, describing its bacterial diversity at the genus taxonomic level. The bacterial populations displaying FITC fluorescence values below 10 correspond to non-opsonized cells.

The mixed FACS-pyrosequencing approach presented here could theoretically also be applied to identify fungi, by using fungal-specific fluorescent markers and subsequent sequencing of PCR-amplified fungal ITS or 28S rRNA regions (Ghannoumet al., 2010). In addition, an RNA-binding fluorophore like pyronin can be used to quantify, separate and sequence-identify transcriptionally active bacteria (Peris-Bondia et al., 2011; D'Auria et al., 2013). In our saliva samples, 31-43% of bacteria appeared to be marked by pyronin (n=6), suggesting that a large portion of organisms in the oral cavity can be viable but transient or inactive. Finally, micro-organisms cell counts can be used to accurately calculate bacterial and fungal load, which can be related to the body fluid chemical and biological components. That way, features of the immune system response can be associated with the microbial composition and density, providing insights about functioning of the immune system and suggesting potential biomarkers of health and disease conditions.

In conclusion, the FACS-pyrosequencing approach appears to be a consistent method to measure Ig-coating levels in oral samples, although the degree of bacterial aggregation in biofilm samples may make it less reliable than saliva. In addition, salivary components may vary in concentration through time due to circadian rythms, feeding and oral hygiene, among others. Thus, I have next applied the methodology to unravel the potential role of salivary Ig-coating in dental caries, by measuring Ig-coating levels in saliva for healthy and diseased individuals through time within a 24-hour period and with a larger sample size (10 individuals per group, at 4 timepoints). In addition, I have related those Ig-coating levels to salivary Ig levels and bacterial load, as well as identifying the bacterial composition in the Ig-bound and Ig-free fractions.

PART B. Ig-Coating through Time and Dental Caries

Total Ig-coating levels in saliva were estimated by using a fluorescent marker that bound to both human IgA, IgG and IgM. The proportion of bacteria coated with IgA,IgG or IgM at 0.5, 6, 12 and 24h after toothbrushing was 70.8, 45.1, 48.8, 61.3% (average 56.5%) in caries-active subjects (>2 active cavities at the moment of sampling, average DMF index= 0.33) and 71.8, 74.8, 73.3 and 70.2% (average 72.5%) for caries-free (DMF index= 0) individuals (n=10 in both groups). Thus, Ig-coating levels were constantly high for caries-free individuals, and more variable for caries-active individuals. Diseased individuals, compared to caries-free, showed normalopsonizationlevels right after toothbrushing, but significantly lower levels at 6 and 12 hours of biofilm formation (p=0.02and 0.05, respectively, Mann-Whitney U-test). At 24 hours of dental plaque formation, caries-active individuals had lower levels of Ig-coating, but the difference was not statistically significant (Figure 4.8).

In agreement with available data (Wilson M, 2005), IgA was the most concentrated immunoglobulin in human saliva, being at one order of magnitude higher levels than IgG, and two orders of magnitude higher than IgM (Figure 4.8). There was a significant change in IgA levels through time in caries free (p=0,034, Friedman's test), but not in caries-active individuals (p=0,34). The difference was due to higher levels in the two morning samples (0.5 and 24h after toothbrushing). There were no significant differences in IgG and IgM concentrations through time in both caries-free and caries-active individuals. IgA was found at higher levels in caries-free individuals compared to caries-bearing individuals at all timepoints, but the difference was significant only at 0 hours (p=0.008, Mann-Whitney U-test).IgG showed higher mean concentrations in diseased individuals at all timepoints, but the difference was not significant. IgM showed no difference between the two groups. There was no correlation

between the proportion of Ig-coated bacteria and Ig levels (p>0.1 at all timepoints, Spearman's Rho coefficient), indicating that the higher levels of opsonization in healthy individuals can not be due solely to higher IgA concentration.

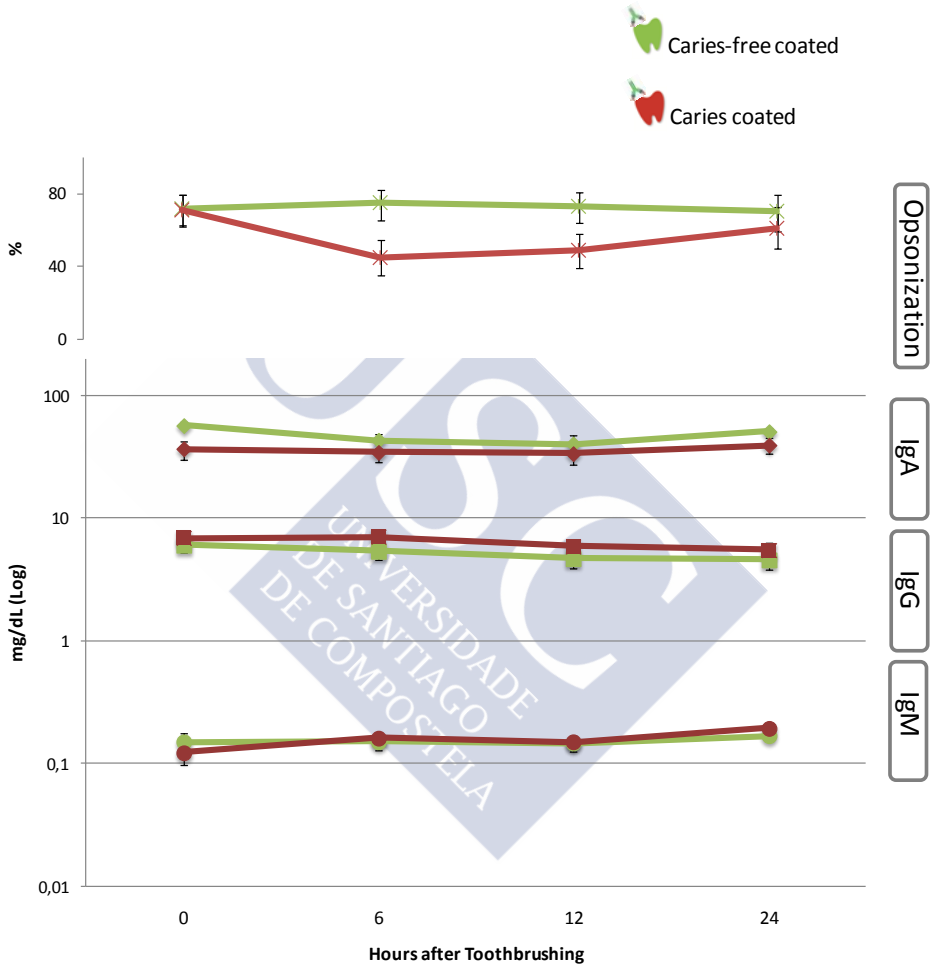

Figure 4.8.Proportion of Ig-coated bacteria in individuals with active caries (red) and in those who had never suffered the disease (green). The lower panels indicatethe temporal variation in salivary immunoglobulin concentrations.Data show means and standard errors for 10 individuals on each group.

Bacterial cell densities per milliliter, as estimated by qPCR, appeared to increase at night (12 h samples) in all donors (Figure 4.9), suggesting that the lower salivary flow at night may increase microbial load until normal salivary levels are restored. This is in agreement with the reported changes in salivary flow through the day due to circadian rhythms (Dawes C, 1972; Papagerakis S et al., 2014).There was a positive correlation between bacterial load and Ig-coating levels at 6 and 12 hours ($p=0.009$ and 0.06, respectively; Spearman's Rho coefficient). Bacterial density was higher at all times in caries-free individuals (p-values were 0.076, 0.009, 0.076 and 0.028 at the four time points; Mann-Whitney U-test), which displayed a 10-fold higher bacterial load than patients with caries lesions (Figure 4.9). Thus, the higher Ig-coating levels in caries-free individuals is not due to lower microbial loads and must be a combination of higher IgA levels and a more effective immune recognition of the microbiota composition present in healthy individuals. In agreement with this, when correlation coefficients were calculated between bacterial load and salivary Ig levels, significant correlations were found only for caries-free individuals, both for IgA ($p=0.015$, linear regression correlation estimate (ce)=-36953.3), IgG ($p=0.0012$, ce=-190641.6) and IgM ($p=0.017$, ce=-7876389.5), and for their respective pair combinations ($p<0.05$ in all cases) 30 minutes after toothbrushing. On the contrary, the absence of significant correlations between bacterial density and Ig levels in caries-bearing patients suggests that the immune system of these individuals has a diminished response to bacterial stimulation.

In addition, this efficiency could be influenced, among other factors, by the degree of bacterial aggregation in saliva and by the specific antigenic composition of microbial populations.

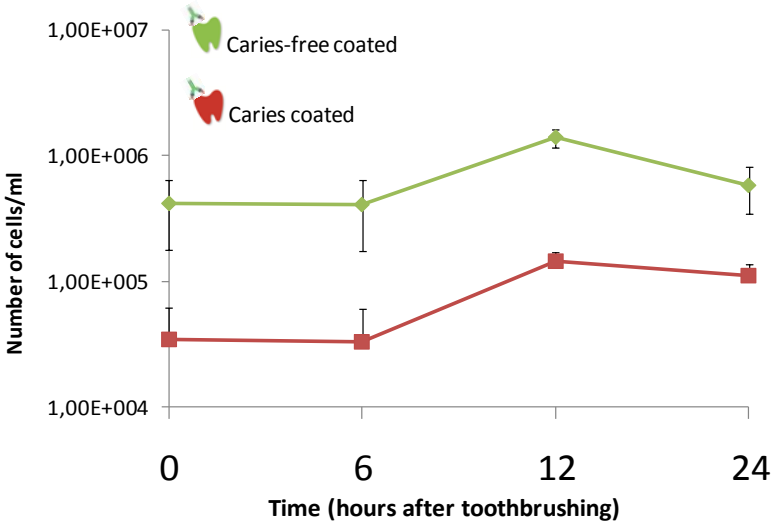

Figure 4.9. Salivary bacterial load through time, as estimated by qPCR. Comparison between caries-free subjects (green line) and subjects with active caries (red line) at 0.5, 6, 12 and 24 hours after toothbrushing. Data show means and standard errors of 5 individuals per group.

When the taxonomic composition was studied by pyrosequencing of the sorted bacteria, it was found that the frequency of many bacterial genera in the Ig-opsonized and non-opsonized fractions was different, suggesting a particular affinity of the antibody for some microorganisms. For instance, data for saliva samples from individual CA021 show that the most common genera in the IgA-opsonized fraction were *Propionibacterium*, *Streptococcus*, *Arthrobacter*, *Veillonella* and *Atopostipes*, whereas the latter three were absent in the non-opsonized fraction. This pattern varied substantially between individuals, indicating that the Ig-recognition pattern and/or the microbiota composition is person-specific, as it is suggested by the large degree of inter-individual variation found in oral microbiome studies (HMP Consortium, 2012). However, some general patterns emerge that suggest that both general microbial composition and Ig-coating patterns vary between individuals with and without dental caries

(Figure 4.10). For instance, *Propionibacterium* appears to be present only in caries individuals, being mostly non-opsonized. *Sphingomonas* appears mainly in healthy individuals, and is mostly non-opsonized. Bacterial genera related to oral diseases, like *Prevotella* and *Porphyromonas*, appear at higher frequency in healthy individuals in saliva during the first hours of biofilm formation and at higher frequency in diseased individuals at 12 and 24 h after toothbrushing in both Ig-coated and uncoated fractions. This suggests that diseased individuals have pathogenic bacteria available in saliva for forming a mature pathogenic biofilm. The application of this technique to larger sample sizes will show if these patterns are maintained or whether the immune recognition pattern is entirely individual-specific.

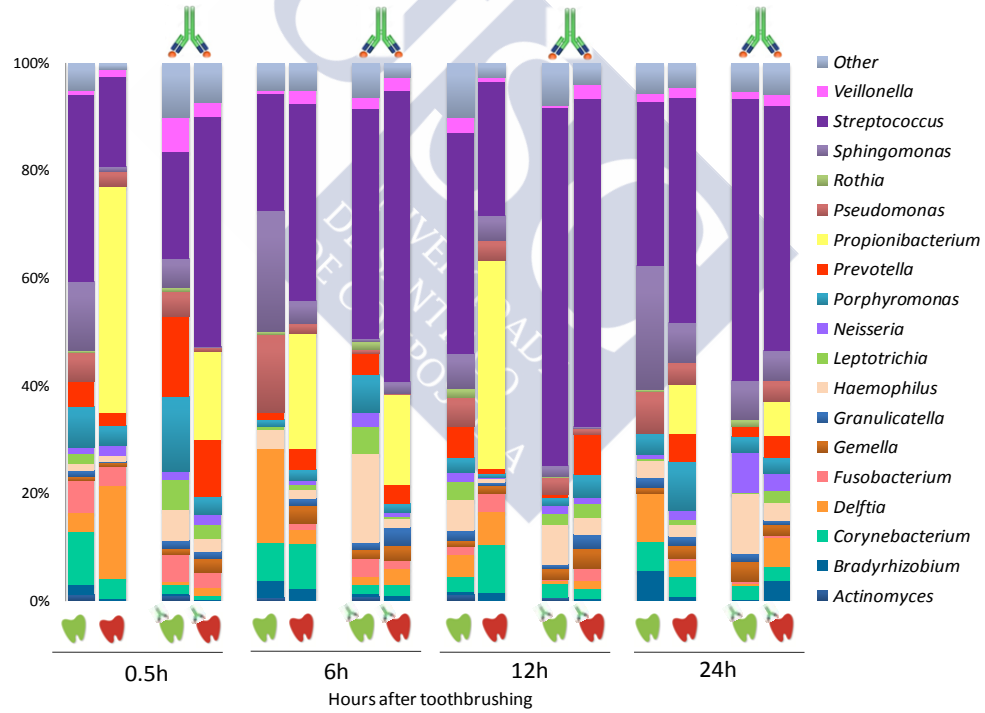

Figure 4.10. Bacterial composition in Ig-coated and uncoated bacteria in human saliva. Unstimulated saliva samples collected 0.5, 6, 12 and 24 h after toothbrushing were stained with a fluorescent marker for IgA-IgG-IgM, and sorted in Ig-coated (Ig icon) and uncoated (red and green teeth) bacteria. Graphs show the bacterial composition, expressed as the median of all individuals, determined by 16S gene pyrosequencing in caries-bearing patients (green tooth) and individuals who had never suffered from dental caries (red tooth).

Differences in bacterial Ig-coating patterns can also be seen when a Principal Coordinates Analysis is performed at the species taxonomic level (i.e. based on sequences clustered at 97% identity). The first two components of the analysis explain 65% of data variability and clearly separate Ig-coated and uncoated samples from caries-free individuals from those corresponding to caries-active individuals (Figure 4.11). In addition, it can be observed that the taxonomic composition of Ig-coated bacteria is very similar at the four time points in caries-free individuals, whereas it is highly variable in patients suffering from the disease. The data therefore indicate that, despite inter-personal variability, the bacterial populations recognized and non-recognized by salivary antibodies are different in individuals with and without dental caries and must be an important factor influencing tooth decay.

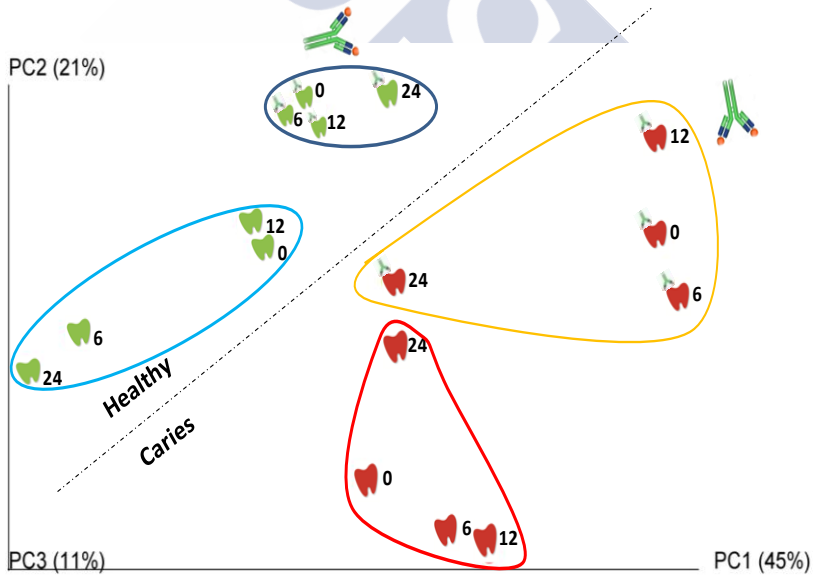

Figure 4.11. Clustering of Ig-coated and uncoated fractions of salivary microbiota through a PCoA analysis. Light and dark blue circles have been drawn to mark the non-opsonized and opsonized fractions of healthy individuals, respectively. Red and orange circles mark the non-opsonized and opsonized fractions of caries-active patients. Numbers indicate sampling time after toothbrushing. Each time-point represents the bacterial composition for 10 individuals as indicated by pyrosequencing of the 16S gene.

4.5. DISCUSSION

The application of the FACS-pyrosequencing technique to healthy and caries-active individuals shown in this chapter strongly indicates a likely contribution of immune competence to the disease, suggesting that therapeutic strategies directed towards improving that immune efficiency should be explored. One of those could involve the use of probiotics to stimulate Ig production (Ashraf R and Shah NP, 2014), although this has been little explored in the oral cavity. Another line of preventive measures could be directed towards breaking down microbial aggregates in saliva (Figures 4.2 and 4.3), therefore liberating bacterial cells and facilitating their recognition and coating by salivary antibodies.

I have shown that the characterization of opsonized and non-opsonized bacterial fractions in caries-free and diseased individuals may unravel the contribution of immune recognition to controlling the populations of oral pathogens. A promising potential of the approach presented here involves the identification of Ig-detected and ignored microbes in healthy and diseased individuals (van der Waaij et al., 2004), ranging from oral diseases (e.g. dental caries or periodontal disease) to gut disorders involving inflammatory responses (e.g. Crohn's disease, ulcerative colitis or irritable bowel syndrome) and even Ig-recognition of tumor cells. Considering immune recognition and opsonization in healthy individuals as ideal, deviations from that balanced microbe-immune interaction can potentially be related to microbial-mediated disorders, and the characterization of individual-specific opsonization profiles can prove fruitful in diagnostic and therapeutic strategies for personalized medicine.

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General Discussion

Simón-Soro A, Mira A. Solving the etiology of dental caries. Trends Microbiol. 2015 Feb;23(2):76-82.

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DE COMPOSTELA

D1. MICROBIOLOGY OF DENTAL CARIES

Classical Koch's postulates contend that a specific microorganism is found to be responsible for an infectious disease when it invades a host, a principle that has been assumed to be correct for most microbial infections. Although the identification of asymptomatic carriers readily showed that the postulates have important limitations and the original formulation has been modified with the introduction of genetic techniques (Fredericks DN and Relman DA, 1996), the principles proposed by Koch have remained a cornerstone in microbiology. Probably due to this, when the sugar-fermenting, acidogenic species *S. mutans* was isolated in the 1920s from carious lesions, it was considered to be the etiological agent of dental caries (Loesche WJ, 1986). Dental caries is considered the most prevalent human disease, affecting 80–90% of the world population (Petersen PE, 2004). In children, it appears to have a fivefold higher prevalence than asthma, which is the second most prevalent disease. For decades, mutans streptococci have been considered the main causative agent of the disease (Loesche WJ, 1975) and most diagnostic, preventive, and therapeutic strategies have been targeted toward this microorganism (see, for example, Plonka KA et al., 2012; Kt S et al., 2013; Islam B et al., 2007). However, other microbial species were also isolated from carious lesions and have been related to the process of tooth decay, including lactobacilli (Badet C and Thebaud NB, 2008) and Bifidobacteria (Mantzourani M et al., 2009). The introduction of molecular approaches to study the human microbiome have revealed that the oral ecosystem is inhabited by hundreds of bacterial species (Bik EM et al., 2010), most of which are considered commensals, and that species regarded as pathogens are frequently found in healthy individuals, although at lower levels than in diseased subjects(Human Microbiome Project Consortium, 2012). An important hurdle in determining the

etiology of tooth decay is that many samples were not taken from the disease site itself but from other, noninvasive samples such as saliva, which, as I have discussed in Chapter 1 of this thesis, does not represent the cariogenic microbiota. However, in a seminal work, Aas JA et al. (2005) obtained over 1200 clones of the 16S rRNA gene from dental plaques and carious lesions at different stages of the disease(Aas JA et al., 2008). This work showed that *S. mutans* could not be PCR amplified in a significant proportion of samples and other bacteria such as*Atopobium*, *Prevotella*, and *Propionibacterium* appeared to be associated with the disease. Recent work added *Scardovia wiggsiae* as a new etiological agent of severe early childhood caries (Tanner AC et al., 2011). In recent years, the use of second-generation sequencing and metagenomic techniques has uncovered an extraordinarily diverse ecosystem where *S. mutans* accounts only for 0.1% of the bacterial community in dental plaque and 0.7–1.6% in carious lesions (Chapter 2). When the DNA of samples from dentin caries was directly sequenced, obviating cloning or PCR techniques, *Veillonella* appeared as the most common genus (Belda-Ferre P et al., 2012), underlining the varying nature of microbial composition in cavities. However, these DNA-based studies may quantify dead, transient, or inactive microorganisms that do not contribute to the disease, inflating estimates of diversity and introducing noise in the analysis (Nyvad B et al., 2013). Thus, the application of high-throughput sequencing to the RNA extracted from oral samples finally provides an opportunity to identify the metatranscriptome; that is, the active microbial composition and expressed genetic repertoire underlying disease initiation and progression.

D2. TARGETING THE CAUSATIVE AGENTS OF DENTAL CARIES

DNA-based studies of microbial diversity in the oral cavity have estimated that the human supragingival dental plaque (the biofilm formed on tooth surfaces) contains between 500 and 700 bacterial species (Bik EM et al., 2010; Aas JA et al., 2008; Belda-Ferre P et al., 2012). These estimates are even higher in saliva, probably because this oral fluid is in contact with all niches in the mouth, reaching values between 1000 and 2000 species in stimulated saliva (Chapter 1, Simón-Soro A et al., 2013b). In carious lesions, however, the number decreases dramatically to 100–200 species-level phylotypes, both in initial, enamel caries lesions and in dentin or deep-dentin cavities (Chapter 2, Simón-Soro A et al., 2013a; Gross EL et al., 2012; Fejerskov O, 2004, Kianoush N et al., 2014), but because these studies are based on PCR amplification of DNA many of the organisms detected may be inactive and not contributing to lesion progression. The recent RNA-based data (Chapter 3) identify bacteria that are actively involved in translation processes, narrowing the list of caries-related organisms to 40–160 per sample, which are presumably those active in individual cavities. It has been assumed for years that the bacteria involved in the disease should also be present in saliva, which has been the preferred oral sample collected in etiological and epidemiological studies of dental caries due to its noninvasive nature (see, for instance, Nasidze I et al., 2009; Rudney JD et al., 2009). However, when saliva, dental plaque, and carious lesions from the same individuals are analyzed, it is readily observed that saliva is not representative of the bacterial diversity located at the disease site (Figure D1). The microbial composition of the enamel lesion under study (orange circle) appears to be dominated by *Veillonella*, *Fusobacterium*, and *Porphyromonas*, whereas in the saliva sample (blue, outer circle) from the same individual *Streptococcus*, *Neisseria*, and *Prevotella* are the genera found at the

highest proportions. This is in agreement with other studies that strongly suggest that saliva samples are not appropriate for studying the microbiology of oral diseases (Simón-Soro A et al., 2013b; Ling Z et al., 2010). Although the dental plaque samples (green circle) are more similar in bacterial composition to that found in their respective carious lesions, several genera decrease and other increase in proportion in the cavity, as a consequence of the more specialized niche. Thus, even dental plaque will not accurately show the bacterial communities responsible for dental caries and the data strongly recommend the use of carious lesion samples with RNA-based approaches (pink, inner circle) to determine the active etiological agents of the disease.

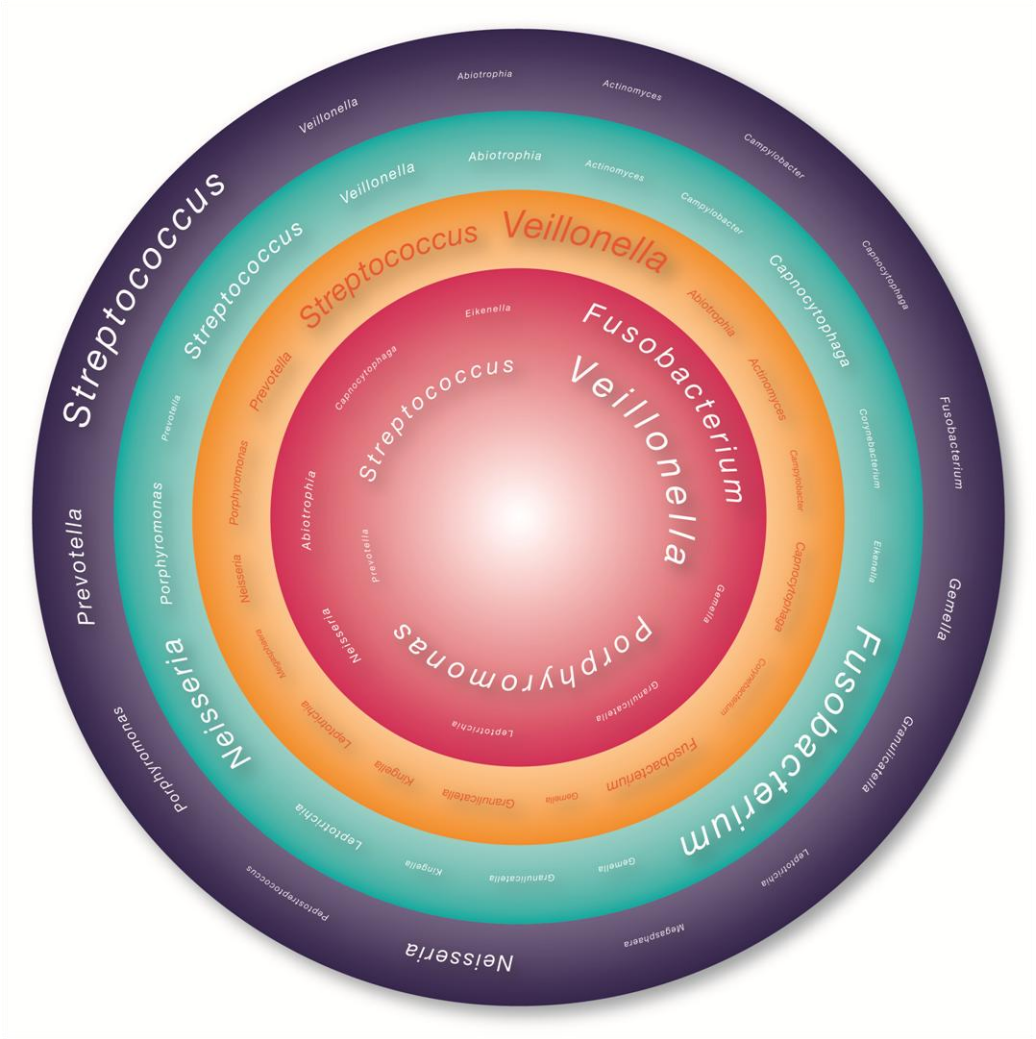

Figure D1. Bacterial composition of different oral samples from the same individual. Each ring corresponds to DNA extracted from drooling saliva, dental plaque from sound surfaces, and an enamel carious lesion, as well as from RNA from the same lesion (inner circle). Font size is related to the proportion of each taxonomic group in the sample.

D3. THE ACTIVE MICROBIOTA OF CAVITIES

The first RNA-based metatranscriptomic study on the surface of teeth (Benítez-Páez A et al., 2014) studied the active microbial communities in oral biofilms before and after a meal, identifying the bacteria that increase their activity after food ingestion, with the premise that these organisms may be involved in sugar fermentation and acid formation. Metatranscriptomic data indicate that the active microbiota is a subset of the total microbial composition in oral biofilms (Duran-Pinedo AE et al., 2014) but is still extraordinarily diverse. In addition, the RNA-based estimates of diversity indicate that different microbial consortia are formed in the dental plaque of different individuals. Thus, determining the active microbiota in carious lesions may finally unravel the elusive etiology of the disease, paving the way for diagnostic and preventive tools.

The first RNA-based estimate of bacterial diversity in cavities is presented in Chapter 3, putatively representing the microbial consortia that are actively contributing to the disease. This approach shows an average of eight active genera at a presence of over 1% in both enamel and dentin lesions. However, each lesion appears to harbor a different combination of bacteria. Except for one case (dominated by *Lactobacillus*), all of the other samples showed diverse bacterial compositions that varied dramatically between individuals, even within the same carious lesion type (Figure 3.4. Chapter 3). The estimated bacterial diversity was lowest for enamel carious lesions, with an average of 177 species-level phylotypes per sample, and highest for 'open' dentin cavities that were exposed to the oral cavity, which had an average of 251 species per sample. This suggests that open dentin cavities have an input

of microorganisms from saliva, even if the biofilm formed on top of the lesion is eliminated from the sample, as was the case here. 'Hidden' dentin cavities, which have almost no contact with the oral cavity except for a minimal lesion through the enamel, had an estimated number of species-level phylotypes of 201 (Simón-Soro A et al., 2014). The observation that multiple species are detected by their RNA in carious lesions unequivocally demonstrates that they are alive and supports the concept that consortia formed by multiple microorganisms act collectively to initiate and expand the cavity. It has been shown that several oral species can act synergistically to increase their pathogenic effect (Murray JL et al., 2014). In a fascinating example of metabolite cross-feeding, *Streptococcus gordonii* was shown to increase the virulence of *Aggregatibacter actinomycetemcomitans* in an animal abscess model (Ramsey MM et al., 2011). Mixed cultures of *S. mutans* and *Veillonella alcalescens* were also found to produce higher acid levels than biofilms containing only one of these species (Noorda WD et al., 1998). In addition, *Veillonella* may favor acid-producing bacteria in caries through nitrate reduction (Doel JJ et al., 2005), given that low concentrations of nitrite killed several cariogenic organisms. Finally, it must be remembered that not only bacterial interactions but also bacterial–fungal associations can be vital for promoting virulence in disease-associated consortia (Harriott MM and Noverr MC, 2011). For instance, *S. gordonii* modulates biofilm formation in *Candida albicans* and *Candida* load influenced oral bacterial diversity and antibiotic resistance (reviewed in Krom BP et al., 2014). Also, viruses may play a vital role in shaping microbial populations (Pride DT et al., 2012), but this has been poorly studied in oral environments (Pride DT et al., 2012).

In the putative list of caries-associated bacteria revealed by this metatranscriptomic approach, *S. mutans* accounts for 0.02% of the active microorganisms in hidden dentin cavities, 0.48% in open dentin cavities, and 0.73% in enamel carious lesions (Chapter 3, Simón-Soro A et al., 2014). Thus,

although there is substantial evidence that *S. mutans* is associated with caries risk, other species clearly arise as main players in the microbial community, including *Veillonella*, *Rothia*, and *Leptotrichia* in enamel caries and *S. sanguinis*, *Atopobium*, *Schlegelella*, *Pseudoramibacter* and *Lactobacillus* in dentin caries. Some of these bacteria are poorly characterized, as exemplified by the genus *Schlegelella*, in which the 16S rRNA sequences identified in this and other studies (Lim SM et al., 2011) indicate that this caries-associated oral microbe is a different species from the only two isolated organisms in this genus that are currently described, both in non-human niches. The polymicrobial nature of carious lesions implies that animal models are probably not representative of human oral disease, especially in cases where single bacterial species are inoculated in the animal (Xu JS et al., 2014; Pekkala E et al., 2002).

A revealing aspect of RNA-based studies is that the composition of active bacteria in initial, enamel lesions appears to be different from that found in more advanced dentin cavities. This observation holds even in cases where enamel and dentin cavities from the same tooth were sampled and analyzed. A remarkable example is shown in Figure D2, where the hidden dentin cavity of a molar tooth had high frequencies of *Neisseria*, *Lactobacillus*, *Megasphaera*, and *Rothia*, whereas a non-cavitated enamel caries lesion had high frequencies of *Haemophilus* and *Gemella*. In addition, the streptococci were dominated by *S. sanguinis* in the dentin cavity, while *Streptococcus mitis* was at significantly higher levels in the enamel caries lesion, which also showed a higher streptococcal diversity (Chapter 3). The metagenomic study described in Chapter 2demonstrated that enamel caries bacteria have an over-representation of dietary sugar-fermenting genes, whereas dentin caries organisms are enriched in genes involved in the metabolism of human-associated glycans (Simón-Soro A et al., 2013a). In addition, enamel caries microorganisms are extremely rich in adhesion molecules whereas the microbial community in dentin caries contains a remarkable arsenal of proteases to degrade dentinary tissue, including

collagenases, dipeptidyl peptidases, serine proteases, glycoproteases, matrix metallopeptidases, and aminopeptidases. The environments that the two cariogenic microbial consortia inhabit are also reflected in the most common stress genes: oxidative stress genes in both tissues, acidic stress genes in enamel lesions, and osmotic stress genes in dentin cavities (Simón-Soro A et al., 2013a). Thus, the data clearly demonstrate that caries microbiology is tissue dependent and does not a have a unique etiology, which has important consequences for disease prevention.

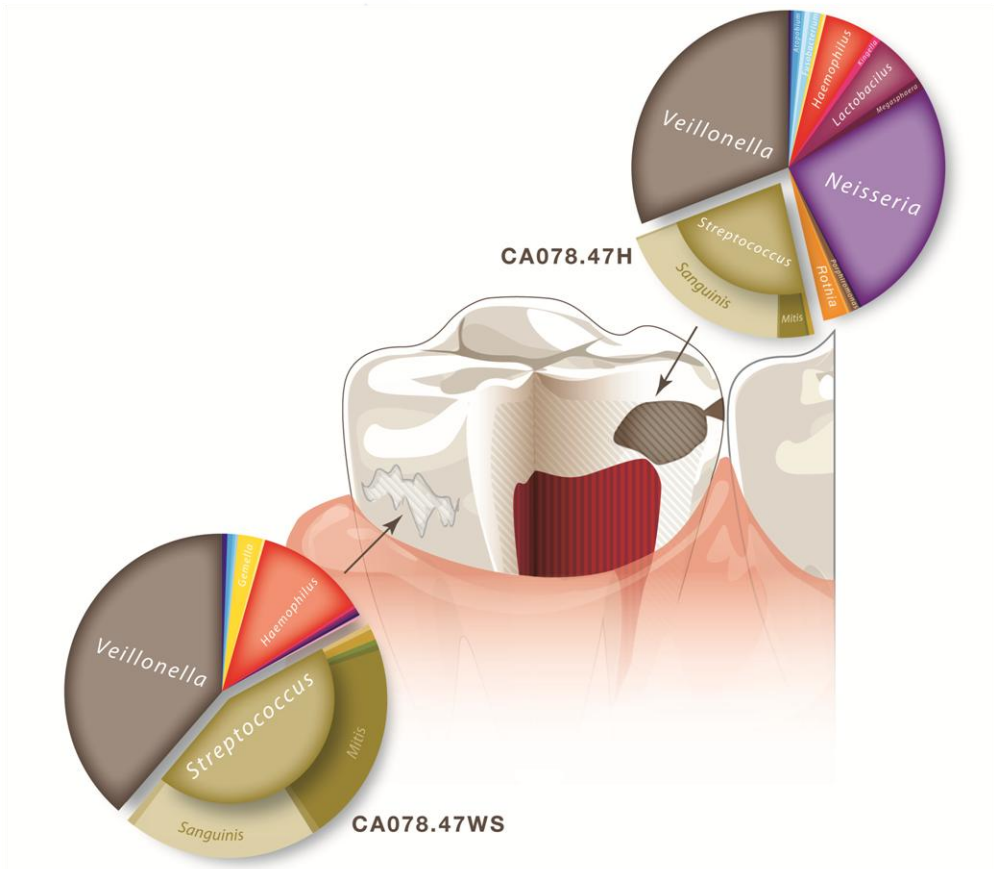

Figure D2.The bacterial composition of caries is tissue dependent. A sample from an initial, white-spot enamel caries (WS) and a hidden dentin lesion were taken from the same tooth (the second molar from the fourth quadrant). The lack of contact between the two lesions was assessed radiographically. The active, RNA-based bacterial taxonomy was obtained by pyrosequencing of long PCR products encompassing hypervariable regions V1 through V4.

D4. IMMUNE SYSTEM AND CARIES

Enamel is the only inert tissue in the oral cavity (and one of the very few in the human body). Thus, it is the only oral niche without a mucosal immune response. Perhaps for this reason, there is limited information about the potential role of the immune system in protecting from, or facilitating dental caries, contrarily to periodontal disease where the immune response to specific periodontal pathogens and the chain of immune events leading to disease have been thoroughly studied (Costalonga M and Herzberg MC, 2014). The work presented in Chapter 4 of this thesis demonstrates that saliva plays a fundamental role in immune protection against cariogenic bacteria. This is reflected in the significantly higher levels of Ig-coating in healthy individuals compared to patients with a history of caries, as well as in a different pattern of microbial recognition by antibodies. In healthy individuals, bacteria ignored by the immune response are most likely non-cariogenic, whereas those escaping opsonization in caries-active patients probably include cariogenic organisms. Thus, I believe that bacterial species which are uncoated by immunoglobulins in diseased individuals but recognized in healthy individuals could be indicative of caries etiology. In any case, if we consider the pattern of microbial Ig-coating in caries-free individuals as the reference "gold standard", significant deviations from this blueprint could be indicative of immune unbalance and an immune-related tendency to develop the disease. A difficulty in interpreting these data, however, comes from the high degree of inter-individual variability in the microbial Ig-recognition profile, which makes it difficult to define general patterns associated to health or disease. In addition, the level of different immunoglobulins in saliva appeared to vary between the two groups. These, together with differences in antimicrobial peptides between healthy and caries-bearing individuals (Malcolm J et al., 2014) suggest that the measurement of

immune-related molecules in saliva could be used as effective biomarkers of caries risk (Guo L and Shi W, 2013).

It is worth underlining that Ig levels and Ig-coating patterns changed through time over a 24-hour period. This suggests that there is a fine-tuning of the immune response. Overall, the data suggest that the immune system plays a vital role in determining the composition of dental plaque and the relative presence of potential pathogens, and this function should be further studied and considered as an important pillar of caries risk. Dental caries is clearly a multifactorial disease, where environmental (e.g. diet), microbiological and host factors play a function in determining the disease. The latter include the immune system as a cornerstone, together with genetic (Zeng et al. 2014) and physico-chemical features of the oral cavity. The study of the human microbiome as a whole and of the oral microbiome in particular has been biased towards studying the taxonomic composition of different samples in health and disease. However, those microbial species interact with the host and are adapted to the special environmental characteristics, among which the immune system exerts one of the most intense selective pressures (Ley RE et al., 2008). Thus, I believe that an imbalance or impairment in the crosstalk between the immune system and the oral microbiota implies a clear tendency to develop caries, and new strategies should be directed at recovering immune competence and balance in diseased individuals. Given that microorganisms do not appear to be planctonic in saliva (se Figures 4.2 and 4.3 in Chapter 4), another possibility could be the physical or chemical break of bacterial aggregates, in order to facilitate the recognition and opsonization of pathogenic organisms by salivary immunoglobulins.

D5. PREVENTING TOOTH DECAY

The fact that caries-associated bacterial consortia vary at different stages of disease progression and are dissimilar in different individuals suggests that the same functions may be performed by different sets of microorganisms. This appears to be a general trend in metagenomic studies of the human microbiome, where radically different taxonomic compositions give rise to surprisingly similar functional profiles (Vaishampayan PA et al., 2010). Thus, even if determining the etiology of a microbe-mediated disease could be considered the first step in designing preventive strategies, knowing the specific microbial composition will not suffice to understand and combat the disease. Initial hypotheses about the underlying microbiology of oral diseases, which focused on determining the specific causative agents, gave way to ecology-based propositions where the disease is seen as the output of a skewed microbial community due to environmental changes (Marsh PD, 2003). If the microbial composition of carious lesions is so variable and the combinations of possible consortia so numerous (Chapters2 and 3), is it relevant to know a list of cariogenic players? The ecological hypothesis of dental caries considers that focusing on the metabolic output of microbial communities can be the clue to understanding and controlling the disease, regardless of the microorganisms involved in the process (Takahashi N and Nyvad B, 2011), and different *omic* approaches have been initiated to understand the holistic functional output of dental plaque (Nyvad B et al., 2013; Takahashi N et al., 2010). From an applied viewpoint, I believe that the detection of disease-associated metabolic profiles would be more informative than microbial composition in predicting risk of oral diseases and could have potential diagnostic value.

Thus, the change in paradigm in the etiology of tooth decay must be translated to appropriate therapies (Fejerskov O, 2004). Given the polymicrobial nature of dental caries, we predict that diagnostic and preventive strategies

directed toward specific bacterial species will not be universally effective. These include diagnostic kits of caries risk assessment, which are traditionally focused on culture counts of mutans streptococci and lactobacilli (Plonka KA et al., 2012) whereas the data show that the former are found at extremely low proportions even within cavities and the latter are found only in several dentin cavities and not in initial, enamel caries lesions (Chapters2 and 3), confirming that this species is not involved in the initiation of caries (Jiang W et al., 2014). Regarding preventive or therapeutic approaches, passive immunization strategies such as the use of synthetic antibodies directed against *S. mutans*-specific antigens (Ma JK, 1998) are predicted to be inefficient given the multispecies nature of caries initiation. In addition, the aggregated nature of salivary microorganisms observed in Chapter 4 may also impede the proper Ig-recognition and coating of cariogenic organisms. Active immunization strategies (i.e., a caries vaccine) are being explored mainly against mutans streptococci antigens (Kt S et al., 2013; Zang S, 2014) and even the multispecies approach that was later proposed (Mira A, 2007) may not suffice if a key, universal bacterial repertoire is not present in caries development. Thus, future research should focus on intercepting functions related to disease initiation and progression regardless of which is the bacterial player behind the role. Tentative examples of functions to be inhibited in future caries preventive strategies might include quorum sensing and other intercellular communication signals, carbohydrate-fermenting pathways, intermicrobial and microbial–human adhesion compounds, bacterial–immune system crosstalk molecules, and pH-regulating particles. Disruption of adhesion molecules may be a fruitful strategy to either prevent or modulate microbial attachment to the tooth pellicle or between key players in biofilm formation (Wright CJ et al., 2014). This is based on the premise that interference in the development of the oral biofilm caused by impairing the tooth attachment of early colonizers or their ability to recruit other key players during biofilm formation would affect the entire process and avoid the presence of cariogenic or periodontal pathogens. For

instance, *Fusobacterium nucleatum* is a promising candidate given its capability to coaggregate with both early and late colonizers of the oral biofilm (Kolenbrander PE et al., 2014). Controlled (e.g., at subinhibitory levels), limited, or specific use of antibiotic therapy has also been proposed (Rogers GB et al., 2010).

Another alternative to modulate plaque development without making use of an antimicrobial strategy is the use of probiotics. The introduction of strains specific to the biofilm could theoretically modify key microbial interactions or buffer cariogenic effects, favoring a healthy, balanced ecosystem. However, current clinical trials with probiotic treatment have not been particularly effective in reducing caries rates (Cagetti, M.G. et al., 2013). A reason for this can be related to the use of single species in probiotic approaches. Not only pathogens act in groups: balance-promoting commensals probably need multispecies clusters to their facilitate settling on the biofilm and to provide beneficial effects. Perhaps for this reason, in the human gut, fecal transplantations have been shown to be effective in treating *Clostridium difficile* infections, colitis, and irritable bowel syndrome, suggesting that an already-formed community settles more effectively in the recipient niche and has a higher probability of conferring a positive effect (Austin M et al., 2014). However, the practical application of oral transplants would have important drawbacks in daily use, ranging from lack of reproducibility to social rejection of patients or lack of control in the dose and composition of the administered material. A potential solution could be the identification of microbial species that may form stable, cooperative consortia in healthy individuals and that could be used to design probiotic complexes with preventive purposes. In addition to these controlled oral transplants, prebiotics – substances that promote the growth of specific microbial consortia – are a promising option but have unfortunately been only modestly explored (Devine DA and Marsh PD, 2009). Future metatranscriptomic, proteomic, or metabolomic approaches (Hart TC et al., 2011) may provide clues to identify key molecules

that could interfere with dental caries initiation and progression (Nyvad B et al., 2013) and could circumvent the complex and elusive polymicrobial nature of this and other oral diseases.

D6. POLYMICROBIAL VERSUS INFECTIOUS DISEASE

Apart from dental caries, other oral diseases have also been clearly identified as polymicrobial, including gingivitis, halitosis, and root canal infections (Rôças IN and Siqueira Jr JF, 2012). A well-studied case is periodontitis, where even the initial three-species cluster proposed to underlie disease development (the so-called 'red complex' of periodontal disease) has proved to be insufficient to explain its etiology (Hajishengallis G and Lamont RJ, 2012). An expanding list of polymicrobial diseases is emerging outside the oral cavity, ranging from abscesses to bacterial vaginosis, diarrhea, rhinosinusitis, and chronic infection in the lung of patients with cystic fibrosis (Peters BM et al., 2012). The common detection of pathogens present at low levels in health in most polymicrobial oral diseases strongly suggests that they cannot be considered to have an infectious nature and that the microbial causative agents are better described as pathobionts (Chow J and Mazmanian SK, 2010; Ayres JS et al., 2012). These organisms are therefore resident bacteria with the potential to cause disease, and under balanced conditions the immune system does not have an active response against them (Table D1). From an applied viewpoint, we believe that antimicrobial treatments will be unfruitful in healing or preventing oral polymicrobial diseases and strategies directed toward modulating microbial interactions and/or their functional output should be further developed.

Table D1. Key features of single-species and polymicrobial oral diseases

Etiology	Kind of Disease	Present in Health	Virulent Behaviour	Immune Response	Treatment
Single- species	Infectious	No (invasive)	Pathogenic	Active	Antimicrobial
Polymicrobial	Dysbiosis	Yes (commensal)	Pathobiont	Basal	Restore balance

D7. A MICROBIAL-BASED DEFINITION OF DENTAL CARIES

Based on the results from this Thesis, I propose that dental caries is a dysbiotic polymicrobial disease caused by pathobionts.

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General Conclusions

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In answer to the raised objectives, the current PhD thesis has found the following specific conclusions:

1. Saliva samples are not representative of the bacterial composition found at tooth surfaces or that located at the gingival crevice because they correspond mainly to the tongue or the mucosal microbiota. Given that dental caries is a disease associated with supragingival dental plaque, we propose the use of dental plaque and caries lesions samples to study the microbial etiology of dental caries.

2. Based on pyrosequencing of the 16S gene, the microbial community undergoes a dramatic reduction in the number of species from a healthy dental plaque to a diseased site, especially in initial enamel caries. However, caries lesions are extraordinarily diverse ecosystems where mutans streptococci account for less than 1% of the bacterial community, strongly supporting the idea that dental caries is a polymicrobial disease.

3. Bacterial composition was found to be different in enamel and dentin caries lesions. In addition, the metagenomic study of bacterial-encoded functions in supragingival dental plaque, enamel caries lesions and dentin cavities suggests that dental caries is a tissue-dependent process. The results support a scenario in which pH and diet are determinants of the disease during the degradation of enamel, but in dentin caries lesions, not only acidogenic but also proteolytic –especially collagenolytic- processes are involved.

4. The active bacterial composition of dental caries, as indicated by RNA-based pyrosequencing, is very diverse and changes during caries progression. Different bacterial consortia have been found in caries lesions from different individuals, in different caries lesions from the same individual and even in different, physically unconnected caries

lesions from the same tooth, supporting the non-specific and the ecological hypotheses of caries disease.

5. The combined use of fluorescence-activated cell sorting and pyrosequencing reveals that healthy individuals have significantly higher levels of Ig-coating in saliva, and that the taxonomic composition of Ig-coated bacteria is different from that found in diseased individuals. Bacterial Ig-coating levels, antibody concentrations and bacterial concentration in saliva change through time in a 24 hour period, indicating that the immune response to oral microbiota is a dynamic process. The differences found between healthy and caries individuals indicate that the immune system plays an important role in preventing caries disease.

6. *Streptococcus mutans* has been found at proportions lower than 1% also in RNA samples from enamel and dentin caries lesions. Thus, I propose that vaccination therapies or any other antimicrobial strategies directed against single species will have a limited success in preventing dental diseases.

7. Based on the results from this Thesis, I propose that dental caries is a dysbiotic polymicrobial disease caused by pathobionts.
8. Microbial knowledge of the etiology of dental caries will allow to design more effective strategies to diagnose, prevent and treat the disease. The data in the current thesis support that dental caries is not an infectious disease, suggesting that strategies guided to balance the biased metabolic environment associated with caries instead of species-specific antimicrobial treatments would be more effective in promoting a healthy oral environment.

Annex 1

Resumen Tesis Doctoral

Etiopatogénesis de la Caries Dental: Composición Microbiana, Actividad Funcional e Interacción con el Hospedador.

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R.1. INTRODUCCIÓN

La caries dental está definida como una enfermedad multifactorial que implica la disolución del componente orgánico y la desmineralización del componente inorgánico de los tejidos duros del diente (Llamas R, 2015). La degradación de ambos componentes es consecuencia de la actividad enzimática y la producción de ácido por parte de las bacterias que forman parte de la placa dental.

Se puede considerar la caries desde tres perspectivas diferentes: el diente, el individuo y la población (figura R.1), las cuales afectan al riesgo de desarrollar la enfermedad y abarcan los principales determinantes del proceso de caries (Fejerskov O y Kidd EA, 2008).

La caries es un proceso multifactorial con muchos condicionantes posibles; sin embargo, no todos actúan en cada persona. Para que se desarrolle la enfermedad, son necesarios los microorganismos, así como la suma de otros factores que pueden variar de una persona a otra (Fejerskov O y Kidd EA, 2008; Cuenca E y Baca P, 2005).

El riesgo de caries en un individuo puede variar con el tiempo ya que muchos factores de riesgo son cambiantes. Éstos pueden ser factores físicos y biológicos (Featherstone JD et al., 2003; Hassell TM y Harris EL, 1995) en función del flujo salival y composición, la presencia de un número elevado de bacterias cariogénicas, exposición insuficiente al fluoruro, recesión gingival, componentes inmunológicos o factores genéticos.

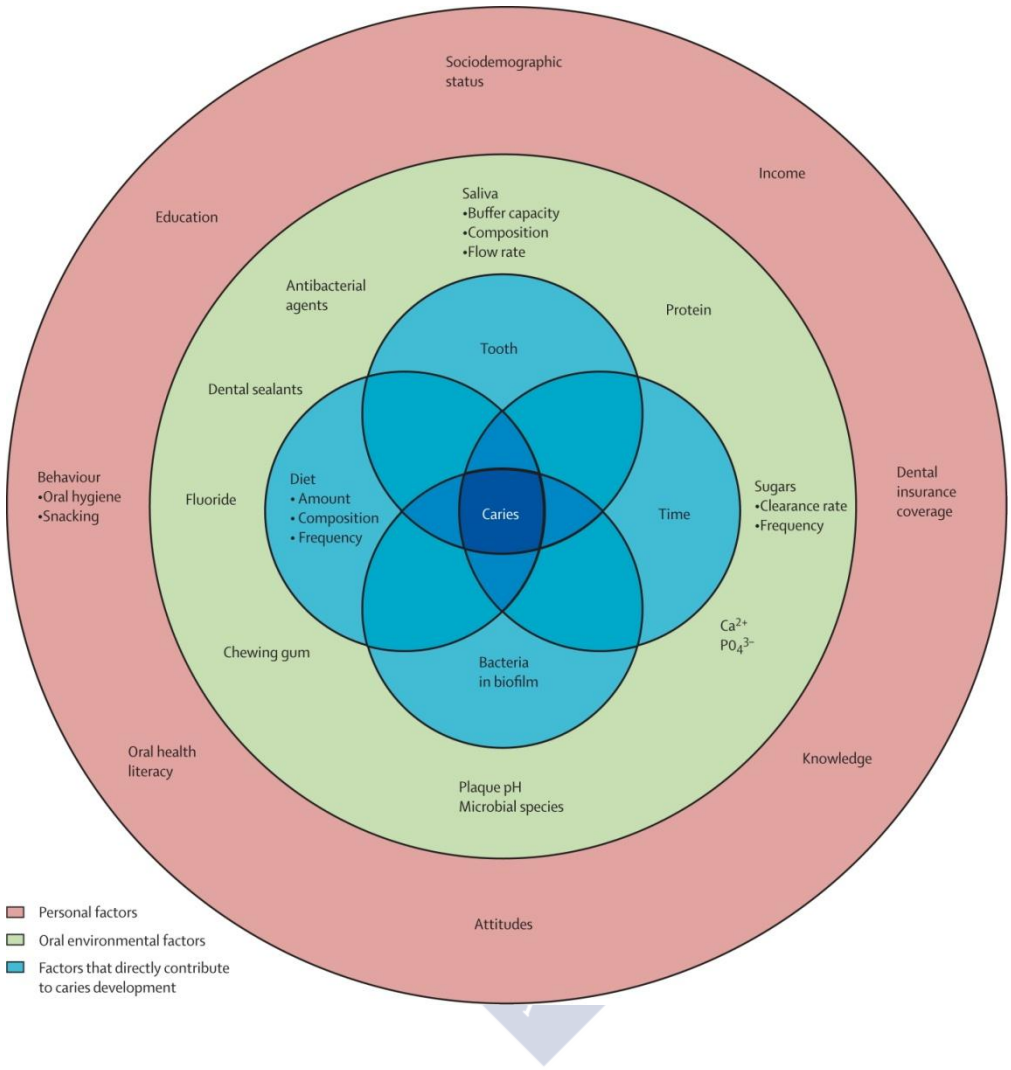

Figura R.1. Ilustración esquemática de los factores determinantes del proceso de caries. Aquellos que actúan a nivel de la superficie del diente se encuentran en el círculo interior (azul). Con el tiempo, un cambio ecológico en la composición y actividad metabólica de la biopelícula (depósito microbiano) puede dar lugar a un desequilibrio entre los minerales del diente y la placa dental, lo que resulta en la formación de una lesión de caries (solapamiento de los dos círculos pequeños). En el anillo intermedio (verde) se incluyen otros factores del ecosistema oral que influyen en el desarrollo de la caries dental. En el anillo (rosa) exterior se enumeran los determinantes más distantes, que influyen en la etiopatogenia de esta enfermedad, a nivel individual y de la población (Diagrama de Keyes modificado. Selwitz RH et al., 2007).

Otros factores son conductuales e incluyen una higiene oral deficiente; los hábitos alimentarios pobres como el consumo elevado de carbohidratos refinados (Touger-Decker R y van Loveren C, 2003),y otros factores relacionados como el estatus social, el nivel educativo o tratamientos preventivos como los selladores de fosas y fisuras también se han relacionado con el riesgo de caries dental.

El término caries dental se utiliza para describir los resultados (signos y síntomas) de una disolución química localizada en la superficie de los dientes causada por agentes metabólicos que se producen en la placa dental que cubre el área afectada. Según el último informe de la OMS, la caries afecta al 60-90% de los escolares (Petersen PE, 2003).

Las lesiones de caries son el resultado de un cambio en la ecología y la actividad metabólica de la biopelícula, mediante el cual se desarrolla un desequilibrio entre los minerales del diente y la placa dental (Marsh PD, 2003). Se caracteriza por una actividad microbiana continua dando como resultado agentes metabólicos en forma de fluctuaciones del pH (García-Godoy F y Hicks MJ, 2008). El resultado acumulativo de numerosas fluctuaciones del pH por meses o años provoca la pérdida de calcio y fosfato, la superficie del esmalte se vuelve porosa y esto puede apreciarse clínicamente en forma de mancha blanca (Figura R.2). Es importante apreciar que aunque muchos resultados metabólicos puedan dar lugar a una lesión de caries detectable, muchas secuencias de eventos metabólicos tienden al equilibrio y por tanto se contrarrestan los fenómenos de desmineralización y remineralización unos a otros, por lo que estos eventos metabólicos deben considerarse intrínsecos a la fisiología de la placa dental (Hicks J et al., 2004).

Cualquier factor que influye en los procesos metabólicos, como la composición y el espesor de la biopelícula, la tasa de secreción de saliva y su composición, la dieta y la concentración de iones de flúor en los fluidos orales, contribuirá a determinar la probabilidad de una pérdida de minerales y la velocidad en la que esto ocurre (Selwitz RH et al., 2007; Featherstone JD, 2004).

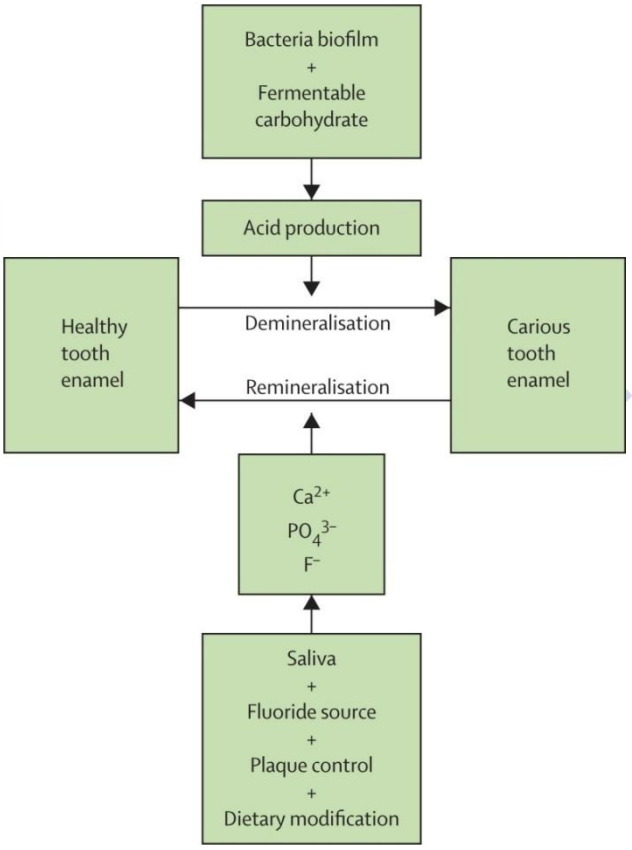

Figura R.2. Diagrama del proceso de caries. Esquema de los diferentes componentes involucrados en el flujo de desmineralización (destrucción) y remineralización (reparación) del tejido dental.

Entre los compuestos inorgánicos destacan el calcio, fosfatos y fluoruros, que son importantes para la precipitación de sales sobre la superficie dentaria, bien en forma de $Ca(H_2PO_4)_2$ a un pH 5 y/o como otras formas de fosfato cálcico cuando el pH se eleva, predominando especialmente $CaHPO_4$. El ion fluoruro tiene fuerte afinidad por Ca^{+2} y las pequeñas cantidades que existen en la saliva actúan fundamentalmente interfiriendo el desarrollo de las lesiones de caries y favoreciendo su remineralización, así como promoviendo la formación de fluorapatita, de mayor resistencia a la desorganización ácida que la hidroxiapatita (Ganss C et al., 2000). Cuando el pH de una solución sobresaturada se reduce gradualmente, el punto en el que la solución se convierte en saturada con respecto al mineral en cuestión se llama el "pH crítico". En este punto se produce la disolución de los cristales de hidroxiapatita, lo que puede causar una cavidad central en los cristales (Axelsson P, 2000).

La caries en esmalte clínicamente visible normalmente aparece por primera vez como lesiones de manchas blancas (Fejerskov O y Kidd EA, 2003), que son pequeñas áreas de desmineralización subsuperficial debajo de la placa dental. Si, por acción de la higiene oral, la biopelícula se retira total o parcialmente, la pérdida de minerales puede detenerse y revertirse por acción de la saliva, que se encuentra sobresaturada con respecto a la hidroxiapatita del esmalte. Esto detiene la progresión de la lesión, e incluso puede dar lugar a la reposición de minerales en la misma superficie del diente.

Los primeros signos de reacciones dentinarias ante lesiones del esmalte que podemos observar en el microscopio es la esclerosis tubular, que se forma en la parte más profunda de la lesión de esmalte. La desmineralización del esmalte aumenta la porosidad y por lo tanto también la permeabilidad del esmalte, lo que provoca que los primeros estímulos leves que inician la reacción

de defensa de la dentina corresponden a la parte más porosa de la lesión de esmalte (Ross MH y Pawlina W, 2007). La esclerosis tubular inicial se observa antes de que la lesión alcance la unión amelodentinaria. Cuando la lesión de esmalte alcanza la dentina, el primer signo observado de desmineralización de la dentina es una decoloración marrón. La absorción de minerales en esmalte y dentina procedentes de la saliva es muy limitada cuando se detiene la enfermedad.

Después de la exposición de la dentina a las bacterias de la cavidad, la parte más superficial se descompondrá por la acción de los ácidos y las enzimas proteolíticas (Fejerskov O y Kidd EA, 2003). Por debajo de esta zona, encontramos invasión de bacterias en los túbulos dentinarios. Si la progresión de la lesión es muy rápida, podemos encontrar túbulos vacíos en la dentina, lo que significa que los procesos de odontoblastos son destruidos sin haber producido la esclerosis tubular. Estos túbulos vacíos son altamente invadidos por bacterias (Love RM y Jenkinson HF, 2002).

La primera reacción en el complejo dentinopulpar es la esclerosis tubular (Kidd EA yFejerskov, 2004). Cuando la lesión de esmalte llega a la unión amelodentinaria, la parte superficial de la dentina se somete a desmineralización que clínicamente se puede ver como una decoloración amarillenta-marrón de los tejidos blandos. La decoloración puede ser resultado de los cambios bioquímicos del colágeno de la dentina debido a la desmineralización. A medida que el proceso continúa, se seguirá con la esclerosis tubular (Fejerskov O, 2008).

El conocimiento de las bacterias de la cavidad oral es uno de los objetivos principales de las investigaciones sobre caries dental. El uso de microscopios y cultivos fue un paso muy importante para ayudar a estudiar esta enfermedad. En 1894, Miller fue el primero en demostrar la invasión bacteriana de los túbulos dentinarios tanto de dentina cariada como no cariada así como en tejido pulpar necrótico, reportando que esta microflora tubular consistía en cocos y bacilos. De esta manera, sentó las bases para determinar la importancia de los ácidos elaborados por bacterias en la producción de la caries. En 1.900 Goadby aisló bacilos Gram positivos de dentina cariada y los denominó *Bacillus necrodentalis*, sugiriendo que participaban en la descalcificación del esmalte y la dentina

Fue JK Clarke, en el año 1.924 quién describió una nueva especie de estreptococo al que denominó *Streptococcus mutans*, aislado a partir de gran parte de muestras de pacientes británicos con caries dental. Russel Bunting describió la caries dental como una enfermedad infecciosa producida por *Bacillus acidophilus* al que años más tarde se denominó *Lactobacillus acidophilus*, siendo éste hoy en día un factor etiológico específico responsable de las etapas tardías de la patología (Gomar-Vercher S, 2013).

Aunque se han detectado más de 700 especies diferentes de bacterias en la boca, el 50% de las mismas no se han cultivado (Aas JA et al., 2005), lo que hace necesario el uso de nuevas tecnologías independientes del cultivo para determinar la diversidad total de este rico ecosistema. La cavidad oral no es un entorno homogéneo para la colonización microbiana, ya que existen diferentes microhábitats, como las mucosas, las diferentes superficies de los dientes, y el surco gingival, donde las comunidades microbianas son distintas (Fejerskov O et al., 1994; Simón-Soro A et al., 2013a).

El uso de técnicas moleculares como la reacción en cadena de la polimerasa (PCR), que tiene por objetivo obtener un gran número de copias de un fragmento concreto de ADN, permitió encontrar nuevas especies de bacterias en la cavidad oral no detectadas mediante cultivo (Benítez-Páez A et al., 2013). Uno de los principales usos de la PCR en la detección de nuevas especies es la amplificación de genes conservados en todas las bacterias que puedan ser usados como "marcadores" taxonómicos. El gen más frecuentemente utilizado en este sentido es el de la subunidad pequeña del ribosoma, o gen 16S del ARN ribosomal (Paster BJ et al., 2001; Bryun R, 2004).

La amplificación de secuencias de genes 16S ARN ribosomal, aparte de identificar nuevas especies no cultivadas hasta hace muy poco tiempo, como el phylum TM7 (He X et al., 2015), y que son bastante frecuentes en la cavidad oral (Paster BJ et al, 2001; Bik EM et al, 2010) permitió detectar en algunas muestras de caries la ausencia de *S. mutans*, lo que supone un gran cambio en la concepción de la enfermedad. Gracias a esta técnica también se observó la presencia de otras bacterias productoras de ácido que pueden ser cariogénicas, como los lactobacilos y bifidobacterias (Aas JA et al., 2005; Mantzourani M et al., 2011).

La pirosecuenciación es una técnica de determinación de las secuencias de ADN a gran escala, aplicable a genomas completos, mediante luminiscencia, capaz de determinar la secuencia de 500 millones de nucleótidos en 24 horas. Gracias a ella se han podido establecer nuevas hipótesis sobre la etiología de la caries y el papel de los microorganismos en la salud y la enfermedad, así como la detección de cientos de especies bacterianas en muestras orales (Belda-Ferre P et al., 2012; Lazarevic V et al., 2009).

Se han asociado distintos microorganismos a lo largo de la historia, describiendo su posible implicación en la caries dental desde la visión de una enfermedad monoespecífica a una polimicrobiana. El principal agente infeccioso asociado como iniciador de la caries dental es *Streptococcus mutans (*Loesche WJ, 1975*).*Esto se debe a las características tanto morfológicas como funcionales y a que puede establecerse en superficie duras como el esmalte dental formando biopelículas. Características asociadas a esta bacteria y que están relacionadas con la enfermedad incluye el que contiene adhesinas en superficie (Krzyściak W et al., 2014), que les permite adherirse al tejido que en el que se desarrolla la caries dental o unirse de forma específica a otros microorganismos (Kolenbrander PE, 2011), así como la presencia de un sistema de transporte de azúcares (Hamada y Slade, 1980). En cuanto a las características funcionales se puede destacar la producción de ácido láctico a partir de azúcares de la dieta (Moye ZD et al., 2014).El metabolismo tiene lugar tanto en un medio neutro como en uno ácido, permaneciendo la actividad si el valor pH es bajo (Köhler et al., 1995), lo que permite crear un ambiente ácido, más extremo y que le confiere ventaja frente a otras bacterias para ocupar el nicho. Además este microorganismo tiene tolerancia a ambientes con pH inferior a 5.5, puede producir polisacáridos intracelulares que le permiten tener un reservorio nutricional en momentos sin alimento; por último, puede producir polisacáridos extracelulares que fomentan el aumento y complejidad de la placa dental, y que además al ser insolubles impiden a la saliva actuar como un elemento protector (Hamada y Slade, 1980).

Otra bacteria ampliamente reportada es *Lactobacillus* spp., descrita como responsable de la progresión de la caries. Principalmente se ha encontrado asociada a lesiones en dentina (Badet C y Thebaud NB, 2008). Este género no tiene capacidad de adherirse a superficies duras, por lo que se encuentra en zonas retentivas, como fosas y fisuras y en cavidades dentales

profundas. Así, estos microorganismos se mantienen en nichos con pH bajo y en zonas de acumulación de placa dental (Beighton y Brailsford, 1998).

En estudios posteriores se ha relacionado *S. sobrinus*y *Veillonella spp*. como especies asociadas a *S. mutans* en el desarrollo de la caries dental (Aas JA et al., 2005).Además se ha visto que existen caries con ausencia de *S. mutans*, por lo que no sería un actor totalmente imprescindible para el desarrollo ni aparición de la enfermedad (Aas et al., 2008).En este caso se ha descrito *Atopobium* asociado a estas lesiones. En función del tipo de caries también se han descrito algunas especies asociadas a este momento del desarrollo. Tanner et al. (2011) propusieron *Scardovia wiggsiae* como agente iniciador de la caries, asociadas a caries infantil de aparición temprana; también se ha descrito *Bifidobacterium spp*. como microorganismos importantes en el primer estadio de la caries (Beighton D et al., 2010). Se ha propuesto además especies del hongo *Candida* como implicados en el desarrollo de esta enfermedad oral, debido a que pueden estar presentes en ambiente acidogénicos. Todo ello refleja el carácter polimicrobiano de la caries dental, y por tanto el avance en las técnicas microbiológicas y moleculares empleadas para su estudio permite entender el complejo ecosistema que abarca esta enfermedad.

Con el fin de explicar la etiología de la caries dental se han descrito principalmente tres hipótesis. La primera es la hipótesis de la placa específica, la cual propone que el efecto patógeno de la placa dental es dependiente del tipo específico de microorganismos residentes en ella. De esta forma, una placa rica en microorganismos Gram positivos y sacarolíticos (fermentables de sacarosa) será una placa tendente a producir caries dental, mientras que una placa con mayor proporción de organismos proteolíticos (que degradan

proteínas) y Gram negativos será una placa periodontopatógena (Loesche WJ, 1992).

Por otro lado, la hipótesis de la placa no específica plantea que todos los microorganismos que colonizan la superficie del diente participan por igual en los procesos patológicos cuando al encontrarse en una cantidad excesiva, son capaces de sobrepasar los mecanismos de defensa que le impone el huésped. Esta teoría le da más importancia a la cantidad de microorganismos que al tipo de ellos (Theilande E, 1986).

Por último, la hipótesis ecológica de la placa sostiene que las bacterias asociadas con la enfermedad pueden estar presentes también en los sitios sanos, pero en niveles tan bajos, que no son clínicamente relevantes. La enfermedad sería el resultado de los cambios ocurridos en el balance de la microbiota que reside en la placa, como consecuencia de la alteración de las condiciones medioambientales locales. Esta teoría se complementa con la teoría mixta ecológica propuesta por Kleinberg I (2002), que cuestiona a estreptococos del grupo mutans como casi la única bacteria implicada en el proceso y como la más cariogénica (Marsh PD, 1994; Marsh PD 2003; Takahashi N y Nyvad B, 2011).

Los microorganismos que se encuentran en la cavidad oral están íntimamente relacionados con los componentes del sistema inmune del hospedador. La inmunidad oral es el sistema de equilibrio que tiene el cuerpo humano para evaluar los microorganismos que se encuentran en los distintos tejidos orales, y las fluctuaciones debido a agresiones externas producidas ya que la boca es una vía de entrada e intercambio con el ambiente. Gracias a la alimentación y a otros factores, existen fluctuaciones constantes del entorno

que deben ser evaluadas y debidamente controladas por el sistema inmune oral, que evitará el paso de sustancias nocivas y microorganismos catalogados por nuestro mecanismo de defensa como potencialmente peligrosos, previniendo enfermedades del organismo.

Los tejidos con capacidad inmunológica en la boca son principalmente los de la mucosa oral, sobre todo en el reconocimiento del entorno y producción de metabolitos reactivos que liberan al medio oral contenidos en la saliva (Feller L et al., 2013; Hovav AH, 2014). Otros tejidos inmunitarios son las glándulas salivares, productoras de las inmunoglobulinas, siendo la IgA el principal componente para este cometido (Carpenter GH, 2013).

Existen distintos componentes del sistema inmune que pueden estar relacionados con la aparición y desarrollo de la caries dental, debido a la interacción y control del ecosistema microbiano establecido en la cavidad oral. Estos pueden ser producidos por la mucosa y liberados para formar parte de los componentes de la saliva, como es el caso de las defensinas humanas (alfa-defensinas 1-3 y beta-defensinas 1-3); las proteinas antimicrobianas (LL-37, catelicidina, calprotectina, lactoferrina), además de las inmunoglobulinas secretadas en saliva (sIgA), y producidas en mucosa (IgA, IgG e IgM).Todos estos metabolitos son necesarios para dar respuesta de forma basal a la presencia de los microorganismos y sus fluctuaciones. Además, cambian sus concentraciones en función al ritmo circadiano del individuo y a lo largo de la vida y de los cambios que se producen en los tejidos que los producen (Castalonga M y Herzberg MC, 2014).

Otro compuesto de la saliva potencialmente relacionado con la caries es la urea, pues se trata de una sustancia orgánica producto final del metabolismo proteico (Carpenter GH, 2013), que cuando se metaboliza puede liberar amonio, produciendo toxicidad sobre las bacterias y acción tampón sobre el pH. La saliva también contiene calcio y fósforo, cuyas cantidades se han relacionado con el riesgo de caries (Feller L et al., 2013).

En cuanto a los tejidos en los que se produce la caries, el esmalte no tiene capacidad inmunológica para poder testar y reaccionar frente a la degradación y colonización de los microorganismos, debido a su carácter inerte. Por tanto, únicamente es la película adquirida formada por compuestos de la saliva y de la encía la que podría tener capacidad de influir sobre la composición microbiana de la placa (Zaura E et al., 2014). Es en el complejo dentinopulpar, una vez pasada la barrera amelodentinaria, cuando el diente es capaz de reconocer las bacterias y activar la inmunidad innata y posteriormente la adquirida con el fin de proteger la pulpa y así salvaguardar la vitalidad dental (Farges JC, 2009).

El mayor problema conceptual que hemos encontrado con respecto a la etiología de la caries se relaciona con el supuesto carácter infeccioso de la caries dental, lo cual se basa en los influyentes postulados de Koch que se considera deben seguir las enfermedades causadas por microorganismos: (1) la bacteria debe encontrarse en el individuo enfermo, pero estar ausente en el sano; (2) el agente patógeno debe estar relacionado a esa enfermedad y no estar involucrado en otra, ni siquiera de manera comensal; (3) el agente patógeno debe ser aislado en cultivo de la zona infectada del individuo enfermo; (4) el microorganismo debe producir la enfermedad en un animal inoculando el

patógeno; y (5) el microorganismo debe ser aislado de las lesiones de los animales inoculados (Fredericks DN y Relman DA, 1996). La caries dental se ha descrito a lo largo de la historia como una enfermedad infecciosa. En principio se relacionó con *Streptococcus mutans* como agente patógeno causante de la enfermedad, pero debido al aislamiento de varias especies potencialmente acidogénicas y a los avances tecnológicos se ha propuesto una etiología polimicrobiana, con numerosas especies presentes en distintos momentos de la progresión de la caries, y en función de los tejidos afectados y las características del ecosistema en cada etapa. La presente Tesis trata de arrojar luz, gracias al uso de técnicas de secuenciación de segunda generación, sobre los agentes etiológicos de la caries dental y el papel de éstos en el proceso completo de la formación de la enfermedad.

Un factor fundamental que predispone al desarrollo de caries dental es obviamente de carácter microbiológico, pero además se encuentran relacionadas las características del hospedador, por lo que cada individuo puede tener unos factores inherentes, tanto genéticos como inmunológicos, que marcan su riesgo de caries y pueden predisponer o favorecer que exista un microbioma oral sano o con tendencia a la enfermedad (Costalonga M y Herzberg MC, 2014). Esta Tesis abarca la interacción del ecosistema microbiano en pacientes con y sin caries, para evaluar con qué herramientas cuenta el hospedador para detectar, interaccionar y detener la caries dental, desde el inicio de la misma en esmalte hasta la afectación pulpar.

La toma de muestras para el estudio de la caries dental se ha basado principalmente en la saliva (McInnes Py Cutting M, 2010), debido a que es el fluido oral que envuelve todos los tejidos, se asume que es reproducible en distintos pacientes, y por su facilidad y accesibilidad en el muestreo (Yang F et

al., 2012). Debido a que en estos últimos años se han desarrollado técnicas moleculares como las técnicas de secuenciación masiva que permiten ver con mayor detalle el contenido microbiano de las muestras, ahora podemos estudiar la composición microbiana de muestras nicho-específicas (Aas JA et al., 2005; Zaura E et al., 2009). En la presente Tesis, se propone, mediante una aproximación taxonómica en relación con los principales microorganismos causantes de la caries dental y usando nuevas metodologías, la toma de muestras más adecuada en estudios etiológicos y/o epidemiológicos de caries dental.

Las aproximaciones metodológicas clásicas como el cultivo han permitido, a lo largo del último siglo, conocer alguna de las bacterias que se encuentran presentes en lesiones de caries, como *Streptococcus mutans* (Clarke JK, 1924), o *Lactobacillus* spp. (Bunting RW, 1937). Posteriormente, gracias a las técnicas independientes de cultivo como la clonación de productos de PCR se pudieron relacionar otros microorganismos que no se podían obtener por técnicas tradicionales de aislamiento, abriendo más la visión en torno a los agentes involucrados, como es el caso de *Atopobium* (Dewhirst FE et al., 2001) o *Bifidobacterium* spp. (Beighton D et al., 2010).Sin embargo, aunque la clonación supone un enorme avance respecto al sesgo del cultivo, los estudios desarrollados por esta aproximación proporcionan típicamente una media de unos 50 clones, lo cual es insuficiente para describir todo el complejo ecosistema de la caries dental durante su desarrollo. En esta Tesis, se han usado técnicas de secuenciación de segunda generación, lo que ha proporcionado la obtención de miles de secuencias por muestra. Esto nos permite abarcar por primera vez la mayor parte de la diversidad que se encuentra presente en relación a la enfermedad.

R.2. OBJETIVOS, METODOLOGÍA Y RESULTADOS

R.2.1. Geografía microbiana de la cavidad oral

En el capítulo se estudian topográficamente distintos micronichos de la cavidad oral, con el objetivo de conocer cada ecosistema con los microorganismos que lo componen. Se ha llevado a cabo mediante aproximaciones moleculares, que permiten comprender con profundidad qué bacterias son específicas de cada zona oral. Además, se ha utilizado esta información para determinar la muestra que mejor explica cada microambiente y su uso para estudios sobre etiopatogenia y/o epidemiológicos. Se han tomado muestras de cada superficie de los dientes individuales y surcos gingivales a partir de dos voluntarios sanos (112 muestras por donante), así como muestras del dorso de la lengua, saliva no estimulada y estimulada de los mismos donantes. Se extrajo el ADN de 67 muestras seleccionadas de cada donante y el gen 16S ARN ribosomal se amplificó por PCR.Se secuenciaron dichos productos de PCR por medio de la pirosecuenciación (454 ROCHE Titanium), obteniendo una media de 2700 secuencias por muestra, que fueron taxonómicamente asignadas para obtener un mapa geográfico de la diversidad bacteriana en cada ubicación oral. Los datos muestran diferencias considerables en la composición bacteriana entre los dientes en diferentes lugares intraorales y entre las superficies del mismo diente (Figura R3).

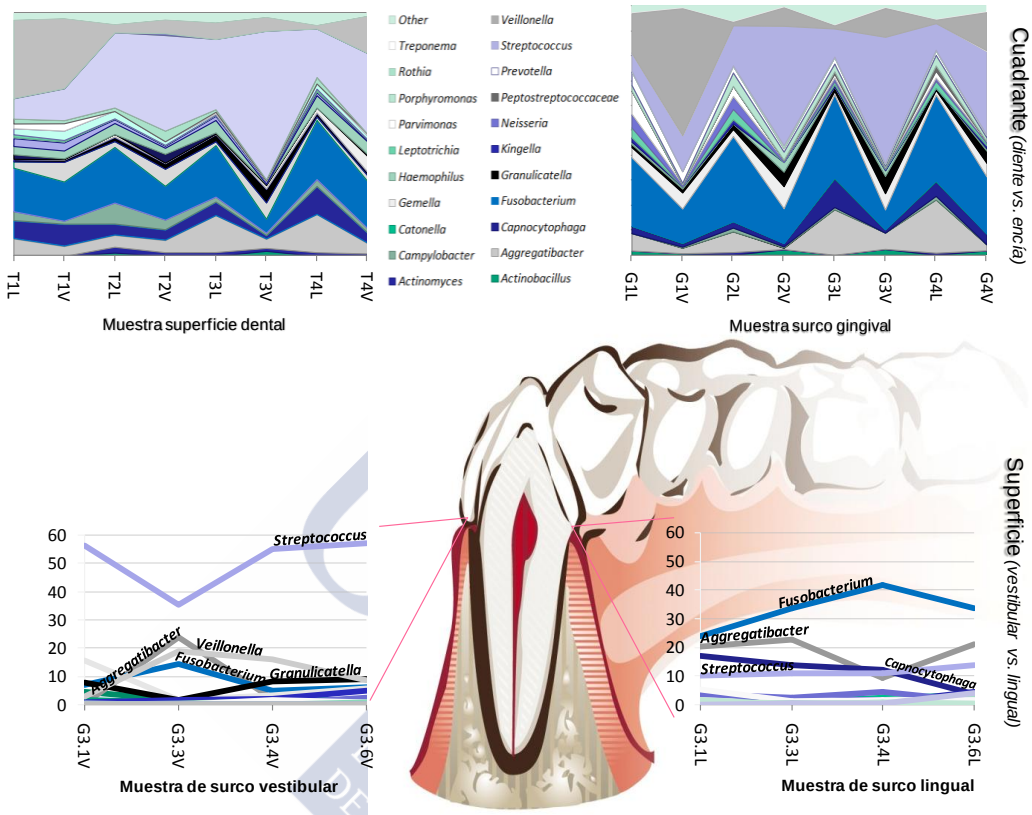

Figura R.3. Composición bacteriana a través de los cuadrantes (paneles superiores) y a lo largo de la arcada dentro de un cuadrante determinado (paneles inferiores). Los paneles superiores muestran la proporción promedio de bacterias en las superficies de los dientes (gráfico de la izquierda) y surcos gingivales (gráfico de la derecha) en los cuatro cuadrantes. Los códigos de muestra indican si se trata de dientes (T) o encías (G), seguido por el cuadrante (números del 1 al 4); V o L se refiere a superficies vestibular (bucal) y lingual (palatino), respectivamente. Los paneles inferiores muestran la variación en la composición bacteriana gingival para sitios del cuadrante 3 vestibular (izquierda) y lingual (derecha). El primer número en cada muestra se refiere al número de cuadrante y el segundo lugar al diente, incluyendo primer incisivo (diente 1), canino (diente 3), el primer premolar (diente 4) y primer molar (diente 6). V = vestibular; L = lingual.

Se observaron las diferencias más pronunciadas en los incisivos y caninos, donde se encontraron géneros como *Streptococcus* al 40-70% en la superficie vestibular, pero estaban casi ausentes en la superficie lingual. Las muestras de saliva, especialmente la no estimulada, no eran representativos de la placa supra y subgingival en los dos individuos analizados. A tenor de los

resultados, las muestras de saliva no son representativas de la composición bacteriana en las superficies dentales o las que se encuentran en el surco gingival porque corresponden principalmente a la lengua o la microbiota de la mucosa. Dado que la caries dental es una enfermedad asociada con placa dental supragingival, proponemos el uso de placa dental y muestras directamente de lesiones caries para el estudio de la etiología microbiana de la caries dental. Esta es por tanto la aproximación que se ha seguido en los capítulos dedicados a la etiología de la caries.

Aunque el conocimiento de los microorganismos presentes en la caries dental es un eslabón necesario para comprender la causa y progresión de la enfermedad, mediante el estudio de la taxonomía no podemos comprender qué papel juega cada uno de los integrantes de este complejo ecosistema. En la presente Tesis, he abordado mediante la metagenómica y metatranscriptómica, no sólo la etiología sino la funcionalidad de los microorganismos presentes en el inicio y progresión de la lesión, a lo largo de la destrucción de los tejidos dentales hasta alcanzar la pulpa dental.

R.2.2. Hipótesis tejido-dependiente de la caries dental

En el segundo capítulo he abordado el estudio de la etiología de la caries mediante un estudio taxonómico y funcional de las lesiones y la placa. La comprensión actual de la caries dental considera esta enfermedad una desmineralización de los tejidos del diente debido al ácido producido por microorganismos mediante la fermentación de azúcares de la dieta. Con el objetivo de identificar la composición bacteriana basada en ADN, se han usado lesiones de caries en las diferentes etapas de la progresión de la enfermedad. Muestras de placa dental supragingival (n=4), lesiones de caries no cavitadas

del esmalte (n= 7) y muestras de lesiones de caries de dentina (n= 11) fueron recogidas de 12 individuos. El material genético fue extraído y utilizado para amplificar el gen del 16S ADN ribosomal. Los productos de PCR de 500 pares de bases de media resultantes fueron secuenciados mediante pirosecuenciación (454-ROCHE titanium) y las secuencias obtenidas fueron utilizadas para determinar la composición bacteriana. Por otra parte, para el estudio por metagenómica directa, se extrajo el ADN y se secuenció en su totalidad, para abordar el estudio de todos los genes presentes en las muestras de placa dental de pacientes sin caries (n=2), placa dental de pacientes con caries recogidas en superficies sanas (n=2), material careado de lesión de esmalte no cavitado (n=1), material de lesiones de dentina (n=1) y dentina profunda (n=1). Posteriormente se abordó el estudio de las secuencias mediante análisis bioinformático para determinar la composición taxonómica y funcional de las comunidades bacterianas.

En este capítulo se presenta el primer análisis metagenómico de las funciones presentes en las comunidades bacterianas en las diferentes etapas del desarrollo de caries, con el objetivo de determinar si la composición bacteriana y perfil bioquímico son específicos para el tejido afectado. Los datos muestran que la composición microbiana en la etapa inicial, que afectan el esmalte de la caries, es diferente a la encontrada en etapas posteriores ($p < 0.002$, Unifrac distance test), y presenta una diversidad cinco veces menor que la placa dental de las superficies de los dientes sanos. Aunque la proporción relativa de *S. mutans* aumentó de 0,12% en la placa dental a 0,72% en la caries de esmalte, *S. mitis* y *S. sanguinis* eran los estreptococos dominantes en estas lesiones. *Prevotella* aumentó en cavidades de dentina entre dos y tres veces con relación a su proporción en las lesiones de esmalte y *Lactobacillus* se encontró sólo en cavidades de dentina profundas.

El perfil funcional de las comunidades bacterianas asociadas a la caries (Figura R.4) indican que los genes implicados en la tolerancia al ácido y al estrés, y en la fermentación de azúcares de la dieta están sobrerepresentados sólo en la etapa inicial (caries del esmalte), mientras que otros genes que codifican para la tolerancia al estrés osmótico, así como diferentes proteasas (incluyendo colagenasas, de la familia s*prT* metalopeptidasas y *omega* peptidasas) que permiten la degradación de la dentina se encuentran significativamente sobrerepresentados en las caries de dentina ($p<0,01$, FDR test).

Los resultados apoyan un escenario en el que el pH y la dieta son determinantes de la enfermedad sólo durante la degradación de esmalte, cuando carbohidratos fermentables están disponibles para bacterias acidogénicas, mientras que en tejido dentinario son otros los elementos que permiten la invasión de este tejido gracias a un metabolismo proteolítico. Se propone que la caries es un proceso de etiología diversa, en la cuales las bacterias productoras de ácido son el vehículo para penetrar el esmalte y permitir a los microorganismos degradadores de dentina expandir la cavidad.

Figura R.4. Perfil funcional de la microbiota presente en la placa dental y lesiones de caries en las diferentes etapas de la progresión de la enfermedad. Cada línea representa una categoría funcional, según lo asignado por el sistema SEED (Meyer et al, 2008), pintado con un color que indica si cada función está sobre- o infra-representadas en las diferentes muestras. Cada columna corresponde a los datos de las muestras individuales de la placa dental de los donantes que nunca tuvieron caries (dientes rodeados por un cuadrado azul), placa dental de las superficies de los dientes sanos de los donantes con más de 10 cavidades en el momento del muestreo (dientes rodeados de un cuadrado verde), esmalte (lesiones de manchas blancas) encuadrado en amarillo, dentina (naranja) y dentina profunda (rojo). Se indican diferencias estadísticamente significativas ("OR" en la figura) en las funciones pertinentes entre muestras de caries del esmalte y la dentina.

R.2.3. Metatranscriptómica revela la composición bacteriana activa global en lesiones de caries

En el capítulo 3se profundiza en el estudio de la etiología de la enfermedad. Con el objetivo de identificar la composición bacteriana basada en ARN, que permite conocer los microorganismos *activos* en la muestra, se han usado lesiones metabólicamente activas de caries en las diferentes etapas de progresión de la enfermedad, y así proporcionar una lista de posibles agentes etiológicos de la caries dental. Las lesiones de caries no cavitadas del esmalte (n= 15) y muestras de lesiones de caries de dentina (n= 12) fueron recogidas de 13 individuos. El ARN fue extraído y retrotranscrito a ADNc, el cual fue utilizado para amplificar el gen del 16S ARN ribosomal, mostrando por tanto las bacterias vivas y creciendo de forma activa. Los productos de PCR de 780 pares de bases de media resultantes fueron secuenciados mediante pirosecuenciación (454-ROCHE titanium) y las secuencias obtenidas fueron utilizadas para determinar la composición bacteriana activa de las muestras.

Se obtuvo un promedio de 4.900 lecturas por muestra, dando una visión integral de las comunidades bacterianas activas en las lesiones de caries. Las estimaciones de la diversidad bacteriana indican que la microbiota de cavidades es altamente compleja, pues cada muestra estudiada contenía entre 70 y 400 especies metabólicamente activas. La composición de estos consorcios bacterianos varió entre individuos y entre las lesiones de caries de los mismos individuos. Además, cada una de las lesiones del esmalte y la dentina tenían un consorcio diferente bacteriano (Figura R.5). Los *Lactobacillus* spp. se encontraron casi exclusivamente en las cavidades de dentina. Los estreptococos representaron el 40% de la comunidad activa total de caries del esmalte y 20% en caries de dentina. Sin embargo, *S. mutans* representaba solamente el 0,02-0,73% de la comunidad bacteriana total.

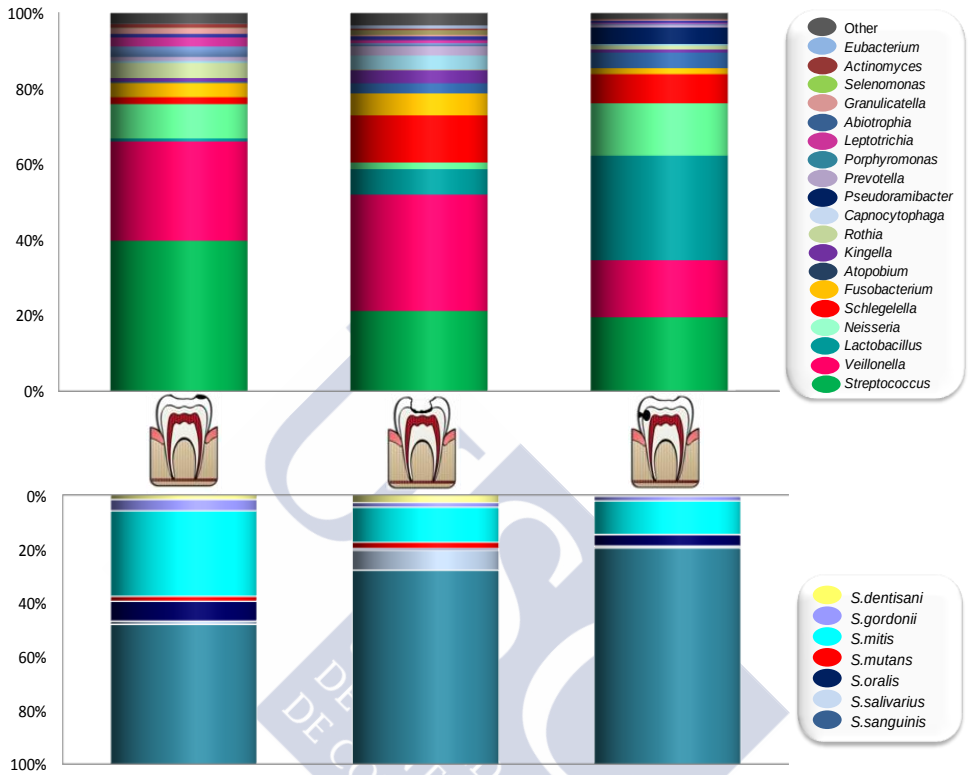

Figura R.5. Composición taxonómica de las bacterias activas en muestras de caries, determinada porpirosecuenciacióndel gen 16S rRNA. Se muestra la proporción de bacterias encontradas por encima del 1% del total (panel superior) y la proporción de las distintas especies de estreptococos (panel inferior);n=15 para caries de esmalte, n=6 para caries de dentina abierta, y n=6 para caries de dentina cerrada.

Los datos indican que la etiología de la caries dental es tejido-dependiente y que la enfermedad tiene un origen claramente polimicrobiano. La baja proporción de *S. mutans* detectado confirma que esta especie es una minoría y cuestiona su importancia como el principal agente etiológico de la caries dental.

Más de un tercio de los microorganismos asociados a seres humanos no han sido cultivados hasta la fecha, obstaculizando la investigación para estudiar las interacciones del microbioma con el sistema inmune, tales como determinar el grado de opsonización de las bacterias en saliva y placa, la especificidad de las especies bacterianas y fúngicas frente a diversos anticuerpos, o la respuesta específica a la carga microbiana en un momento temporal. Idealmente, todo ello se debería estudiar en condiciones de salud frente a enfermedad.

R.2.4. Revelando el reconocimiento microbiano por anticuerpos salivales específicos

El capítulo 4aborda el concepto de interacción entre microbiota y hospedador en salud y caries dental. Hemos combinado el uso de citometría de flujo y pirosecuenciación para describir la composición microbiana de las muestras de saliva de individuos adultos (20-45 años) y su interacción con el sistema inmune mediante el uso de marcadores fluorescentes que son específicos para las diferentes inmunoglobulinas. El marcaje apropiado de células y anticuerpos se comprobó mediante microscopía confocal de fluorescencia. Para descartar el reconocimiento no específico de bacterias por parte de las inmunoglobulinas se usaron anti-Ig de ratón (Figura R.6).

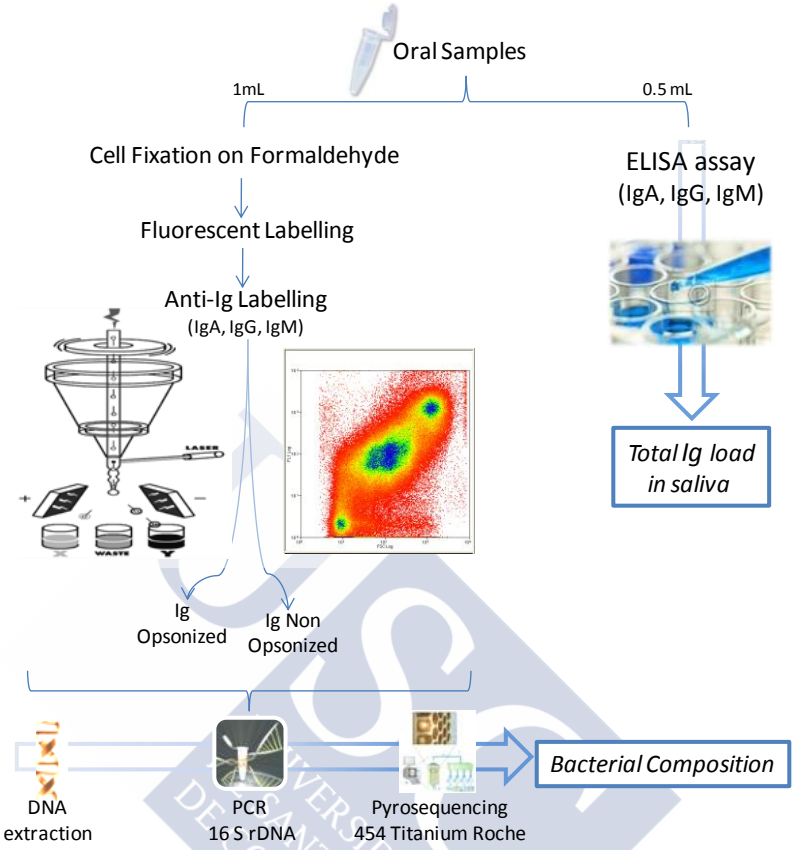

Figura R.6. Flujo de trabajo para el estudio de la interacción de inmunoglobulinas con las bacterias en saliva.

Mediante el uso de la separación celular activada por fluorescencia (FACS, por sus siglas en inglés), las células bacterianas fueron separadas dependiendo de si estaban recubiertas con cualquiera de los tres anticuerpos más frecuentes en la saliva (IgA, IgG e IgM). Una vez separadas las poblaciones de bacterias opsonizadas y no opsonizadas, el ADN de cada población fue extraído, se amplificó por PCR el gen bacteriano 16S ARN ribosomal y se empleó la técnica de pirosecuenciación (ROCHE titanium), con el fin de caracterizar los microorganismos que son ignorados y aquellos que son reconocidos por las

inmunoglobulinas. La proporción de bacterias recubiertas con anticuerpos IgA, IgG o IgM medido en cuatro puntos temporales durante un período de 24 horas (Figura R.7a) fue de 60,4% en sujetos de individuos con caries (> 2 activas en el momento del muestreo) y 83,6% para individuos sanos (n= 10 en ambos grupos). En personas sanas hubo un mayor número de especies bacterianas en la fracción unida a anticuerpos en comparación con la fracción sin recubrimiento, y no se observaron diferencias en este respecto con las personas enfermas. Sin embargo, la frecuencia de muchos géneros bacterianos en la fracción reconocida frente a la no reconocida por anticuerpos es diferente, lo que sugiere una afinidad particular de los anticuerpos por algunos microorganismos.

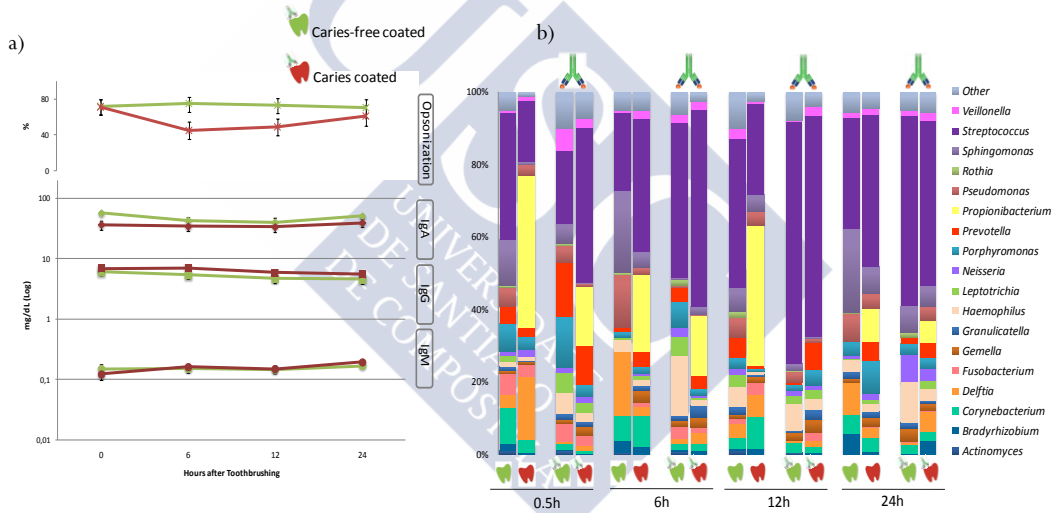

Figura R.7. Proporción de componentes de sistema inmune y bacterias en individuos con caries y sanos. a) Variación temporal en la concentración de inmunoglobulinas salivales (paneles superiores) y proporción correspondiente de bacterias Ig-revestida en individuos con caries activas (rojo) y en aquellos que nunca habían sufrido la enfermedad (verde). Los datos muestran las medias para 10 personas en cada grupo.b) Composición bacteriana en Ig-opsonizada y sin opsonizar en la saliva humana. Las muestras de saliva no estimulada se recolectaron 0, 5, 6, 12 y 24 h después del cepillado, fueron teñidas con un marcador fluorescente para IgA-IgG-IgM y separadas en Ig-opsonizada (icono de Ig) y bacterias sin recubrimiento (dientes rojos y verdes). Los gráficos muestran la composición bacteriana, expresada como la mediana de todos los individuos, determinada por pirosecuenciación del gen 16S en pacientes enfermos (diente rojo) y las personas que nunca han sufrido caries dental (diente verde).

Aunque el perfil taxonómico de reconocimiento fue variable entre individuos, surgen varios patrones. Por ejemplo, a las 24h de formación de la placa dental, en muestras de saliva la frecuencia reconocida de células de *Streptococcus* y *Porphyromonas* fue significativamente mayor en individuos sanos. Otras bacterias como *Leptotrichia* spp., que eran en su mayoría reconocidas en pacientes enfermos, eran bien ignoradas por el sistema inmune o presentes igualmente en ambas fracciones de los individuos sanos (Figura R.7b). La aplicación de la técnica en individuos sanos y con patología revela una importante contribución de la respuesta inmune a la enfermedad. En general, los datos sugieren que el sistema inmune es más competente en individuos libres de caries, lo que indica que tiene un efecto crucial en la generación de la enfermedad. Se concluye por tanto que los niveles de reconocimiento bacteriano, las concentraciones de anticuerpos y la concentración bacteriana cambia en saliva a través del tiempo en un período de 24 horas, lo que sugiere que la respuesta inmune a la microbiota oral es un proceso dinámico. Las diferencias encontradas entre individuos sanos y con caries revelan que el sistema inmune desempeña un papel importante en la prevención de la caries.

R.3. CONCLUSIONES GENERALES

Como conclusión general, en función de los resultados obtenidos para explicar la etiología de la caries (capítulos 1, 2 y 3) y el papel del sistema inmune en el control de la enfermedad (capítulo 4), la aportación de esta Tesis al campo de la cariología intenta explicar la caries dental (Tabla R.1) proponiendo un cambio de concepto. Tal como se ha definido en el pasado, la caries dental es una enfermedad infecciosa producida principalmente por una especie bacteriana patógena (*S. mutans*), la cual es detectada por el sistema

inmune, principalmente IgA, y puede erradicarse mediante el uso de estrategias antimicrobianas.

Tabla R.1. Principales características de enfermedades orales con etiología individual y polimicrobiana.

Etiología	Tipo de patología	Presencia en salud	Virulencia	Respuesta inmune	Tratamiento
Especies únicas	Infecciosa	No (invasiva)	Patogénica	Activa	Antimicrobiano
Polimicrobiana	Disbiosis	Sí (comensal)	Patobionte	Basal	Restablecer el equilibrio

Planteamos una visión diferente de la caries dental basada en cambios en el ecosistema oral. La caries dental está producida por un desequilibrio en la microbiota propia de la placa dental (disbiosis). Algunos microorganismos poseen factores de virulencia capaces de producir el deterioro de los tejidos tanto orgánicos como inorgánicos y la invasión de la dentina. En condiciones favorables a su crecimiento, éstos producen un ecosistema más extremo que les confiere ventaja ecológica frente a otros y desestabilizan el ecosistema oral, tal como sostiene la teoría ecológica de la placa. No existe una respuesta inmune propia contra estos microorganismos, ya que se encuentran tanto en salud como enfermedad, cambiando éstos de proporción y siendo mejor definidos como patobiontes. Por tanto, se propone que el tratamiento más adecuado sería restablecer el equilibrio ecológico, por ejemplo mediante el uso de prebióticos y probióticos, que permitan volver a la situación armónica en la que el nicho oral se regula a sí mismo, y no los tratamientos antimicrobianos tradicionales, dirigidos a especies concretas.

La actual tesis doctoral ha encontrado las siguientes conclusiones específicas:

1. Las muestras de saliva no son representativas de la composición bacteriana en las superficies dentales o la que se encuentra en el surco gingival, ya que según los resultados es similar principalmente a la hallada en lengua o la microbiota de la mucosa. Dado que la caries dental es una enfermedad asociada con placa dental supragingival, proponemos el uso de placa dental y/o muestras de lesiones de caries para el estudio de la etiología microbiana de la caries dental.

2. La comunidad microbiana encontrada mediante pirosecuenciación del gen 16S, sufre una drástica reducción en el número de especies de la placa dental en salud a un sitio de enfermo, especialmente en caries inicial del esmalte. Sin embargo, las lesiones de caries son extraordinariamente diversos ecosistemas donde *Streptococcus mutans* representa menos del 1% de la comunidad bacteriana, apoyando firmemente la idea de la caries dental es una enfermedad polimicrobiana.

3. Se han encontrado diferencias entre lesiones de esmalte y dentina en su composición bacteriana. Además, el estudio de metagenómica de funciones bacterianas en la placa dental supragingival, las lesiones de caries del esmalte y dentina cavidades sugiere que la caries dental es un proceso dependiente de tejido. Los resultados apoyan un escenario en el cual pH y la dieta son determinantes de la enfermedad durante la degradación del esmalte, pero en lesiones de caries de dentina, no sólo acidogénica sino también proteolítica –especialmente colagenolítica- están involucrados en el proceso.

4. La composición bacteriana activa de la caries dental, según lo indicado por pirosecuenciación basado en ARN, es muy diversa y se dan cambios durante la progresión de la caries. Diferentes consorcios bacterianos se

han encontrado en el mismo tipo de lesión de caries de diferentes individuos, en lesiones de caries en distinto estadío de progresión dentro del mismo individuo e incluso entre las lesiones de caries distintas, físicamente no conectados desde el mismo diente, apoyando las hipótesis no específica y la ecológica propuestas para explicar la etiología de la caries.

5. El uso combinado de separación celular por fluorescencia, con citrometría de flujo, y pirosecuenciación revela que las personas sanas tienen niveles significativamente más elevados de opsonización por Ig en la saliva, y que la composición taxonómica de las bacterias opsonizadas es diferente en pacientes sin caries a la encontrada en individuos enfermos. Los niveles bacterianos opsonizados, las concentraciones de anticuerpos y concentración bacteriana cambia en saliva a través del tiempo en un período de 24 horas, lo que indica que la respuesta inmune a la microbiota oral es un proceso dinámico. Las diferencias encontradas entre sanos e individuos con caries indican que el sistema inmune desempeña un papel importante en la prevención de la enfermedad caries.

6. *Streptococcus mutans* se ha encontrado en proporciones inferiores a 1% también en muestras de ARN de las lesiones de caries del esmalte y la dentina. Por lo tanto, propongo que las terapias vacunación u otras estrategias antimicrobianas dirigidas contra una sola especie tendrá un éxito limitado en la prevención de enfermedades dentales.

7. La presente tesis propone por tanto definir la caries dental como una enfermedad disbiótica y polimicrobiana causada por patobiontes.

8. El conocimiento microbiano de la etiología de la caries dental permitirá diseñar estrategias más efectivas para diagnosticar, prevenir y tratar la enfermedad. Los datos en la presente tesis apoyan que la caries dental

no es una enfermedad infecciosa, lo que sugiere que las estrategias guiadas, para equilibrar el ambiente metabólico parcial asociado con caries en lugar de los tratamientos antimicrobianos específicos, serían más eficaces en la promoción de un entorno oral sano.

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Annex 2

Articles Published

RESEARCH REPORTS
Biological

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Microbial Geography of the Oral Cavity

ABSTRACT

We aimed to determine the bacterial diversity of different oral micro-niches and to assess whether saliva and plaque samples are representative of oral microbial composition. We took minute samples from each surface of the individual teeth and gingival crevices of two healthy volunteers (112 samples *per* donor), as well as samples from the tongue dorsum and non-stimulated and stimulated saliva. DNA was extracted from 67 selected samples of each donor, and the 16S rRNA gene was amplified by PCR and pyrosequenced to obtain, on average, over 2,700 reads *per* sample, which were taxonomically assigned to obtain a geographic map of bacterial diversity at each tooth and sulcus location. Analysis of the data shows considerable differences in bacterial composition between teeth at different intra-oral locations and between surfaces of the same tooth. The most pronounced differences were observed in incisors and canines, where genera like *Streptococcus* were found at 40% to 70% on the vestibular surfaces but were almost absent on the lingual sides. Saliva samples, especially non-stimulated saliva, were not representative of supra- and subgingival plaque in the two individuals tested. We suggest that more precise sampling is required for the proper determination of oral microbial composition and to relate that diversity to epidemiological, clinical, and etiological parameters.

KEY WORDS: saliva, massively parallel sequencing, gingival sulcus, dental plaque, sampling, human microbiome.

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INTRODUCTION

It is well-established that the composition of microbial communities varies in different parts of the oral cavity (Segata *et al*., 2012). The tongue, teeth, mucosa, palate, and gingiva have been shown, by both culture and molecular-based approaches, to harbor a distinctive microbiota (Aas *et al*., 2005). Thus, oral environments could themselves be heterogenous in their physico-chemical properties and therefore host different bacterial repertoires. For instance, Kleinberg and Jenkins (1964) measured salivary flow and pH in different parts of teeth and showed clear contrasts in pH values between teeth and even in surfaces from the same teeth which were in close proximity. This classic work showed that the oral cavity is home to multiple micro-niches where not only pH, but also oxygen, temperature, or redox potential, among others, can influence the settling of micro-organisms and risk of disease (Fejerskov *et al*., 1994).

Molecular-based, culture-independent techniques, such as the use of 16S rRNA profiling, have provided important new insights into the diversity of the microbiome within the oral cavity (Crielaard *et al*., 2011). Massively parallel pyrosequencing is an open-ended molecular approach that allows for extensive characterization of microbial populations in a high-throughput, cost-effective manner.

It has been stated that, for gathering complete information on the healthy oral microbiome, microbial samples should be obtained from various ecological niches throughout the oral cavity and from precise and well-characterized disease sites (Nyvad *et al.*, 2013); however, few studies have been published that apply specific sampling for accurate characterization of the different oral micro-niches (Haffajee *et al*., 2009; Zaura *et al*., 2009; HMP, 2012; Segata *et al*., 2012).

Given that many oral microbiology studies are based on pooled samples from different teeth (see, *e.g*., Li *et al*., 2007; Aas *et al*., 2008; Bik *et al*., 2010), it is imperative to characterize differences in microbial composition among specific oral locations within teeth and gingival crevices, since this could severely influence the interpretation of results. Thus, the aim of the present study was to analyze the bacterial diversity in two healthy individual oral cavities at various intra-oral niches by targeted pyrosequencing of the V1-V2-V3 hypervariable regions of the small subunit ribosomal RNA. Sampling locations included different dental surfaces of all teeth and different surfaces of gingival sulcus, across the 4 quadrants. In addition, the bacterial diversity of non-stimulated saliva *vs.* stimulated saliva samples from the same individuals was also evaluated and compared with that from dental and gingival locations to evaluate whether the saliva samples frequently used in etiological and epidemiological studies of oral disorders (Quinque *et al*., 2006) are representative of the microbial diversity at the sites where the disease takes place.

MATERIALS & METHODS

Donor Selection and Sampling Procedure

Two volunteers were selected for sampling. They were men aged 20 to 30 yrs, non-smokers, with 28 teeth excluding third molars, and in good dental and periodontal health [in both, absence of caries (non-cavitated level), DMF = 0, OHI = 0, GI = 1, and CPI = 1 (WHO, 1997)]. They had not been treated with antibiotics in the 6 mos prior to the study nor did they report antecedents of routine use of oral antiseptics. The two donors signed a written informed consent, and the sampling procedure was approved by the Ethics Committee from the DGSP-CSISP (Valencian Health Authority), with reference 10/11/2009.

In each individual, supragingival dental plaque samples were taken, 24 hrs after toothbrushing, from vestibular (buccal) and lingual (palatine) surfaces of 28 teeth, each sample with a different sterile spoon excavator. Teeth were not dried before sampling. From the 56 dental plaque samples collected, 32 were selected for PCR amplification, namely, samples from the first incisor, canine, first premolar, and first molar from each quadrant. Additionally, 56 subgingival samples were taken from the same tooth surfaces, by means of 2 sterile absorbent paperpoints (size 25) *per* sample, passed across each gingival sulcus, avoiding contact with the supragingival dental plaque. Again, 32 samples were selected for PCR amplification. A sample from the tongue dorsum was collected with a sterile spoon excavator across the entire surface, with several repetitive strokes to ensure a representative sample. All the samples were taken in the morning, while the donors sat in a quiet atmosphere. Donors also provided a 2-mL non-stimulated saliva sample 24 hrs after toothbrushing by drooling saliva into a sterile Falcon 50-mL tube. A 2-mL quantity of stimulated saliva was collected immediately after, by the use of a sterile paraffin gum.

DNA Extraction, PCR Amplification, and Pyrosequencing

DNA was extracted separately from each sample by means of the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), following the manufacturer's instructions, with the addition of a lysozyme treatment (Belda-Ferre *et al*., 2012). Thus, no physical lysis treatment was performed, which could influence the diversity detected in the extracted DNA (Kuczynski *et al.*, 2011). A PCR amplification of the 16S rRNA gene was performed with the high-fidelity ABGene DNA polymerase (Thermo Scientific, Epsom, Surrey, United Kingdom) by the use of universal degenerate primers 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 533R (5′-GCCTTGCCAGCCCGCTCAGGC-3′), at an annealing temperature of 52°C and 20 amplification cycles, to minimize PCR amplification bias. Two PCRs were performed *per* sample, and the 2 PCR products were pooled before purification. In 4 gingival samples, a PCR product could not be obtained, and a nested-PCR was performed, in which the PCR product was purified and used as a template for a secondary PCR in which the primers were shifted 3 bp toward the 3′ end and included the pyrosequencing adaptors A and B, following Benitez-Páez *et al*. (2013). The 500-bp PCR products were purified with the Nucleofast PCR purification kit (Macherey-Nagel, Düren, Germany) and further cleaned by AMPure XP beads (Roche, Basel, Switzerland) before pyrosequencing. The final DNA *per* sample was measured by picogreen fluorescence in a Modulus 9200 fluorimeter (Turner Biosystems, Sunnyvale, CA, USA) so samples could be mixed in equimolar amounts. PCR products were pyrosequenced from the forward primer end only by using a GS-FLX sequencer with Titanium chemistry (Roche). One-eighth of a plate was used for each pool of 20 samples, which were amplified with a different forward primer containing a unique 8-bp "barcode."

Sequence Analysis

Reads with an average quality value lower than 20 and/or with more than 4 ambiguities in homopolymeric regions in the first 360 flows were excluded from the analysis. Read ends were trimmed in 10-bp windows if they had a quality value lower than 20. Only reads longer than 200 bp were considered, as well as those without mismatches in the primer region. Chimeric sequences were detected by Mothur (Schloss *et al*., 2009), and 5.4% of the reads were filtered out as potential chimeras. Singletons were not excluded from the analysis. Sequences were assigned to each sample by the 8-bp barcode and analyzed with the Ribosomal Database Project classifier (Cole *et al*., 2009). Each read was taxonomically assigned down to the genus level with an 80% confidence threshold, and reads giving no bacterial hits were excluded from the analysis. All figures and statistical comparisons were performed on samples from each individual separately. To estimate total diversity, we clustered sequences at 97% nucleotide identity over 90% sequence alignment length and obtained rarefaction curves using the RDP pyrosequencing pipeline. Principal coordinates analysis (PCoA) was performed with FastUnifrac (Lozupone *et al*., 2006). The Unifrac analysis compares the 16S-estimated diversity with a phylogenetic approach that takes into account both taxonomically assigned and unassigned reads.

RESULTS

After quality filtering, an average of 2,767 sequences of the 16S rRNA gene was obtained *per* sample (range, 790-6,550), which were assigned at the genus taxonomic level, giving a comparative view of the bacterial diversity and composition among teeth and gingival sites. A summary of these differences in composition can be observed in Fig. 1. The top panels show the average bacterial proportions amplified for lingual and vestibular surfaces from the 4 quadrants, in teeth and gingival sulci. Pronounced differences were observed between vestibular and lingual sites of teeth and gingival sulci, indicating that some bacteria are characteristic for certain sites. For instance, genera like *Streptococcus* were found at 29% to 70% and 23% to 57% on the vestibular surfaces of teeth and sulci, respectively, but were found at lower levels on the lingual surfaces of the same tooth (0% to 51%) or sulcus (5% to 21%) in 97% of the samples. At both teeth and gingival sulci, the 3rd and 4th quadrants had a more similar composition than quadrants 1 and 2, and displayed a higher percentage of *Capnocytophaga* and *Aggregatibacter*. The difference in bacterial composition between the upper and lower arcades was significant for both gingivae and teeth

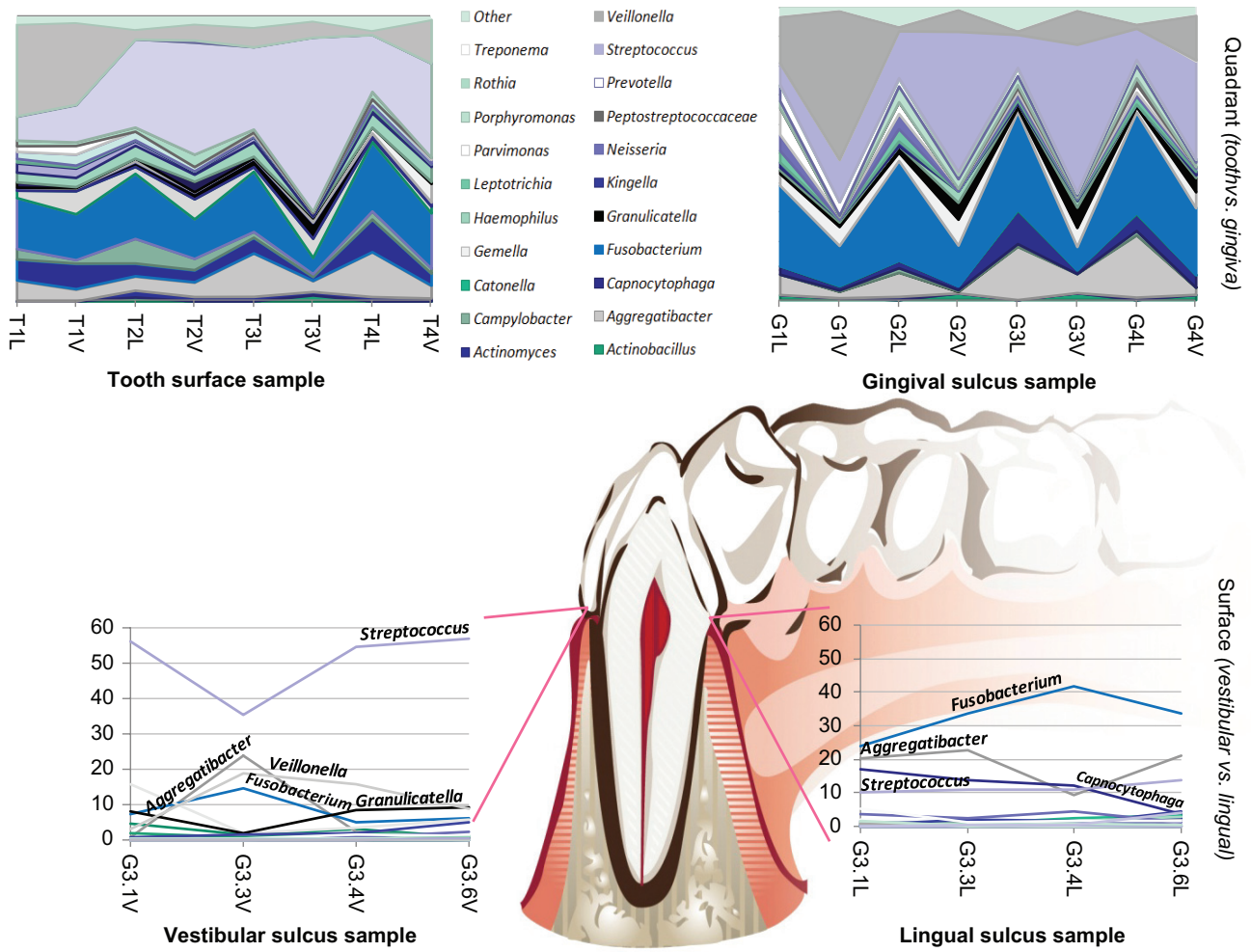

Figure 1. Bacterial composition across quadrants (top panels) and along the arcade within a given quadrant (lower panels). **Top panels** show the average proportions of bacteria at tooth surfaces (left graph) and gingival sites (right graph) at the 4 quadrants. The sample codes indicate whether teeth (T) or gingivae (G) were sampled, followed by the quadrant (numbers 1 through 4); V or L refers to vestibular (buccal) and lingual (palatine) surfaces, respectively. **Lower panels** show the variation in gingival bacterial composition for quadrant 3 at vestibular (left) and lingual (right) sites. First number at each sample refers to the quadrant and second number to the tooth, including first incisor (tooth 1), canine (tooth 3), first premolar (tooth 4), and first molar (teeth 6). V = vestibular; L = lingual. Graphs from all quadrants are available from the authors upon request.

(Unifrac distance test with 1,000 permutations, $p < .001$ in both vestibular and lingual comparisons).

Interestingly, when the compositional pattern along the arches was plotted separately for gingival samples from the vestibular and lingual sites (Fig. 1, lower panels), inverse relationships could be found for several bacteria, mainly involving *Streptococcus*, which appeared to increase in proportion when *Fusobacterium* and *Aggregatibacter* decreased, and *vice versa*. A similar pattern was found along teeth (Appendix Fig.). Both on dental surfaces and in gingival sulci, *Veillonella* was present at a much higher proportion in canines than in incisors, premolars, or molars. In most quadrants, *Streptococcus* was found at higher proportions on vestibular compared with lingual surfaces.

In addition to bacterial composition, diversity was also found to vary considerably at different tissues and sites within tissues. Sequences of the 16S rRNA were clustered at 97% nucleotide identity, which is considered the threshold for species boundaries (Yarza *et al.*, 2008). Thus, each cluster of sequences which is at least 97% similar forms an "operational taxonomic unit" (OTU) that serves to estimate the approximate number of bacterial species. Saliva and tongue samples appeared to harbor a considerably higher number of OTUs than teeth or gingival sulci (Fig. 2). Teeth and gingival samples had a similar level of diversity, and the lingual surface of gingival sulci showed the lowest level of OTUs.

A convenient way to view the similarities among all samples can be provided by a PCoA, where samples are closer to or farther from each other in a multidimensional space, depending on their degree of similarity in bacterial composition. When applied to all samples, the PCoA shows a clear distribution

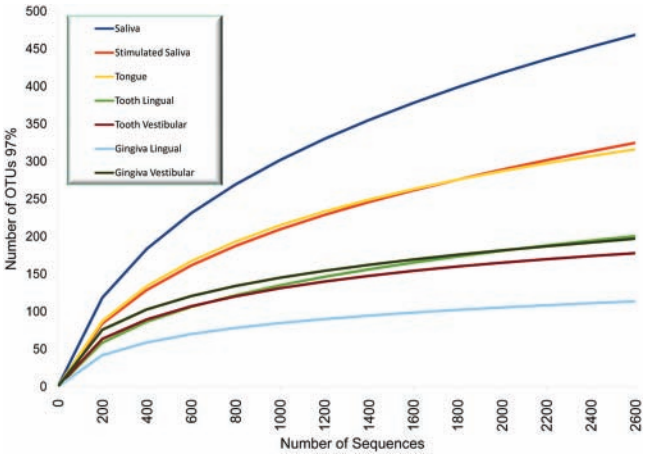

Figure 2. Bacterial diversity in different samples from individual MG01. Rarefaction curves indicate the relationship between sequencing effort and the estimated number of species-level OTUs (reads clustered at 97% sequence similarity). Numbers next to the sample types indicate the estimated number of OTUs at 2,400 16S rRNA reads. Saliva and tongue show the largest diversity. A similar result was found for individual MG02 except for saliva samples, with the stimulated saliva showing larger diversity than the non-stimulated saliva.

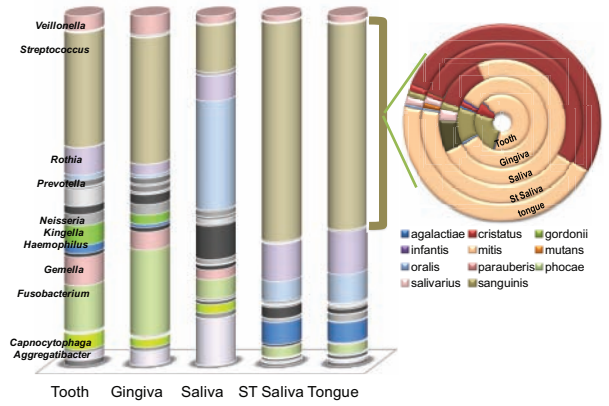

Figure 4. Bacterial composition at the genus level in different oral samples. Columns show the proportion of 16S rRNA sequences obtained from dental, gingival, unstimulated saliva, stimulated saliva, and tongue from individual MG01 (the graph for individual MG02 is available from the authors upon request). Streptococci are the dominant bacteria in most samples, and a ring graph with the species assignments for this genus is shown on the right.

pattern where gingival and dental samples cluster separately (Fig. 3A). In addition, vestibular and lingual samples also appear to cluster together with their own type, showing that each of these niches has a distinct bacterial composition.

Saliva samples taken from the two individuals showed a higher level of diversity compared with the vestibular and lingual areas of both teeth and gingival sites, suggesting that saliva must contain bacteria from other oral niches (Fig. 2). Stimulated saliva showed a lower diversity than non-stimulated saliva in individual MG01 (Fig. 2), but the reverse trend was observed in individual MG02. The latter would be consistent with the removal of bacteria from different oral cavity sites by the mechanical process of paraffin-gum-chewing. However, the PCoA plot showed that the stimulated saliva sample clustered tightly with the tongue sample (Fig. 3B). This was confirmed by the comparative analysis of bacterial composition in saliva, tooth, gingival sulcus, and tongue, which showed a remarkable similarity between tongue dorsum and stimulated saliva (Fig. 4).

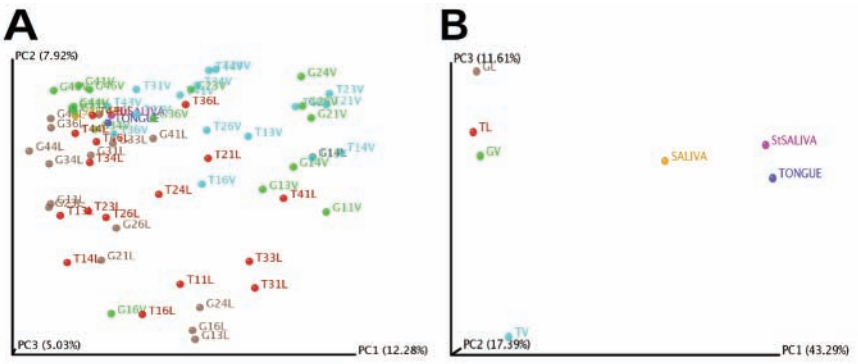

Figure 3. Relationship between gingival and dental samples in terms of bacterial composition. The principal coordinates analysis (PCoA) was performed with Unifrac (Lozupone *et al.*, 2006) and clusters individual samples according to species-level taxonomic composition **(A).** Saliva and tongue samples are included for comparison in a second PCoA in which dental and gingival samples from vestibular and lingual sites were pooled **(B).** Data are for individual MG01. A similar pattern was found in the PCoA for individual MG02.

The PCoA plots showed that non-stimulated saliva appeared to have a microbial composition very different from that of teeth and gingival samples (Fig. 3). The reason for this lack of correlation can be observed in Fig. 4: Some of the most common bacteria in 24-hour supra- and subgingival plaque (particularly *Fusobacterium* and *Veillonella*) were at very low or even absent proportions in saliva; in addition, saliva also showed several bacterial genera at considerable proportions (including *Prevotella, Rothia*, and *Haemophilus)*, which were at very low levels or even virtually absent on teeth and/or gingival sites. This indicates that some bacteria colonizing the tooth and gingival sulcus are not represented in saliva, and that micro-organisms from oral sites other than the teeth or gingivae are present in saliva in considerable proportions.

DISCUSSION

Analysis of the data presented in this article demonstrates the considerable differences in bacterial diversity and composition between individual sites and surfaces of the oral cavity, underlining the extraordinarily diverse set of micro-niches even within the same anatomic location. Particularly striking were the

contrasts in bacterial composition between lingual and vestibular sites, since the proportions of some ubiquitous genera were highly characteristic of the surface sampled (Fig. 1, Appendix Fig.). For instance, *Streptococcus* was found, on average, at 48.2% and 43.8% in vestibular surfaces of teeth and gingival sulci, respectively, whereas it was detected at 23.9% and 12.33% at lingual surfaces. Thus, microbial diversity studies may be strongly biased if only one tooth surface or gingival site is sampled.

The reasons for such variability in microbial composition probably lie in the gradients and variations in physico-chemical features at different locations of the mouth (Kleinberg and Jenkins, 1964). One of these environmental factors affecting bacterial distribution could be oxygen. This is supported by the high proportion of obligate anaerobes like *Fusobacterium* at lingual sites, whereas aerobes or facultative anaerobes like streptococci are highly common at vestibular sites or incisors and canines, which are likely to be exposed to higher levels of oxygen. It is also very likely that pH plays a major role in that distribution pattern, since lingual and vestibular surfaces may have a different buffering effect of saliva. However, the opposite patterns displayed for some bacteria in the lower-panel curves in Fig. 1 also suggest possible antagonistic effects, which have been supported experimentally in the pairs *Fusobacterium-Streptococcus* (He *et al*., 2012) and *Aggregatibacter-Streptococcus* (Whitmore and Lamont, 2011).

We believe that this intra-oral variability in microbial colonization patterns should be taken into account when dental plaque samples are pooled for the assessment of bacterial composition. In addition, the fascinating site-specificity of bacteria observed in the present study may have important implications for oral microbiological studies. Neither stimulated nor non-stimulated saliva was representative of the bacterial diversity, composition, and proportions found at supra- and subgingival sites in the two individuals sampled in this study. Given that the tooth surfaces and the periodontal pockets are the sites where dental caries and periodontal disease take place, the use of saliva as a proxy for bacterial composition at those sites may not provide meaningful correlations between bacterial composition and disease status in epidemiological and etiological studies. Although some evidence has been found between microbial composition of saliva and oral diseases (Yang *et al.*, 2012), a major limitation is that most molecular studies performed thus far are association studies, and the suggested biomarkers have not been tested in a longitudinal clinical study. Therefore, we do not know at present if the observed biomarker candidates are clinically relevant, and neither tests targeting single salivary bacteria nor salivary bacterial tests in combination with clinical parameters have been able to adequately predict the course of caries *in vivo* (Tellez *et al.*, 2012). In addition, some of these studies have found an association between microbiota and disease in plaque samples but not saliva, in both gingivitis and dental caries (Ling *et al.*, 2010; Huang *et al.*, 2011), suggesting that saliva may not be representative of the microbial population at the disease site. Carious cavities, for instance, have been found to harbor a different set of bacterial species than dental plaque at sound surfaces, and the bacterial composition varies among enamel, dentin, and deep dentin samples (Aas *et al*., 2008; Belda-Ferre *et al*., 2012). Thus, including individuals with caries lesions at different stages of disease progression may introduce further 'noise' into the detection of salivary biomarkers for caries. A further complication is given by the use of different saliva-sampling protocols in different laboratories, including paper-points, mouthrinses, and oral swabs (Keijser *et al*., 2008; HMP, 2012).

Exploring microbial diversity in the oral cavity offers the possibility for a better understanding of the role of micro-organisms in health and disease (Takahashi and Nyvad, 2012). However, precise and appropriate sampling must be made to relate health and disease status to microbial profiles. The work presented here is based on data from two individuals, and studies with a larger sample size would be needed to confirm the validity of the observed results. The variability of microbial diversity depending on the oral site sampled is nevertheless clear, and we hope that the present study will help to decide the appropriate sampling strategy in future oral microbiological studies.

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A Tissue-Dependent Hypothesis of Dental Caries

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Key Words

Acidic stress · Dentin caries · Diet · Enamel caries · Metagenomics · Osmotic stress · pH value · Pyrosequencing · *Streptococcus mutans* · 16S rRNA gene

Abstract

Current understanding of dental caries considers this disease a demineralization of the tooth tissues due to the acid produced by sugar-fermenting microorganisms. Thus, caries is considered a diet- and pH-dependent process. We present here the first metagenomic analysis of the bacterial communities present at different stages of caries development, with the aim of determining whether the bacterial composition and biochemical profile are specific to the tissue affected. The data show that microbial composition at the initial, enamel-affecting stage of caries is significantly different from that found at subsequent stages, as well as from dental plaque of sound tooth surfaces. Although the relative proportion of *Streptococcus mutans* increased from 0.12% in dental plaque to 0.72% in enamel caries, *Streptococcus mitis* and *Streptococcus sanguinis* were the dominant streptococci in these lesions. The functional profile of caries-associated bacterial communities indicates that genes involved in acid stress tolerance and dietary sugar fermentation are overrepresented only at the initial stage (enamel caries), whereas other genes coding for osmotic stress tolerance as well as collagenases and other proteases enabling dentin degradation are significantly overrepresented in dentin cavities. The results support a scenario in which pH and diet are determinants of the disease during the degradation of enamel, but in dentin caries lesions not only acidogenic but also proteolytic bacteria are involved. We propose that caries disease is a process of varying etiology, in which acid-producing bacteria are the vehicle to penetrate enamel and allow dentin degrading microorganisms to expand the cavity.

Dental caries is a multifactorial disease caused by microbes and influenced by diet, hygiene habits, tooth shape and strength, saliva-buffering capacity and host immune system, among other factors [ten Cate, 2009]. Cavities are produced by demineralization of the teeth due to low pH when the mineral part of the tissue dissolves due to desaturation, which is moderated by acids [Fejerskov and Manji, 1990]. This would be possible when bacteria accumulate in dental plaque and ferment dietary carbohydrates over a sufficient amount of time, so the acid produced locally cannot be neutralized by the buffering capacity of saliva [Marsh, 1994]. Although the ability of pH lower than a critical value to demineralize enamel is well established [Weatherell et al., 1984; Dawes, 2003], it can only partially explain dentin caries because the mineral component is only a limited portion of dentin tissue. In fact, the two tissues are so different in nature, composition, structure and access to dietary food that explaining caries formation and progression by a single cause may not be feasible. In addition, the etiological factors of dental caries are also unclear. Although initial work related

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the presence of acidogenic mutans streptococci to dental caries [Loesche et al., 1975; Loesche, 1992], other microorganisms like lactobacilli or *Candida* species have been proposed to produce acid and contribute to the disease [Becker et al., 2002; Munson et al., 2004]. This gave rise to a nonspecific plaque hypothesis which sustains that caries can be the result of different acid-producing organisms [Theilade, 1986]. Important evidence showing that the disease is polymicrobial and would therefore not follow the classical Koch's postulates of infectious diseases has recently been provided by molecular methods, showing that mutans streptococci cannot be PCR amplified or hybridize against specific DNA probes in a considerable proportion of cavities [Corby et al., 2005; Aas et al., 2008]. These studies have shown that cavities can be complex ecosystems and suggest that other species like *Atopobium*, *Veillonella* or *Corynebacterium*, which are preferentially found in caries lesions [Belda-Ferre et al., 2012], could also be cariogenic. In fact, it has been proposed that dental caries is not caused by a concrete set of organisms but by a change in the community composition due to external factors which would shift the mineralization balance towards a destructuring of the tooth tissue [Takahashi and Nyvad, 2011]. In all cases, the cause of dental caries as a whole is considered to be the acid produced by fermentation of sugars ingested in the food. In the current manuscript, we study dental caries through a metagenomic approach, presenting data that question this single-cause explanation of the disease. We aim to test the hypothesis that caries, from both a hard tissue and a microbiological perspective, may be considered as a two-step process, namely initiation/demineralization of enamel followed by progression through dentin.

In order to shed light on the causes of dental caries, a complete assessment of microbial diversity composition and function must be performed at different stages of caries progression, and culture-based methods have proven to be insufficient because a large portion of oral bacteria cannot be cultured by classical laboratory methods and growth media [Aas et al., 2005]. Seminal work by Aas et al. [2008] used amplification and cloning of the 16S rRNA gene and subsequent Sanger sequencing to study the microbial diversity in white-spot lesions, dentin caries and advanced cavitated lesions. This pioneering work suggested that the microbial taxonomic composition could change during caries progression, but the low number of sequences obtained by this method (averaging 53 sequences per sample) limits enormously the full description of the diversity in these niches, which can reach several hundred species [Belda-Ferre et al., 2012]. The arrival of second-generation sequencing techniques like Roche pyrosequencing now allows obtaining hundreds or thousands of 16S rRNA sequences per sample, obviating the cloning steps [Keijser et al., 2008]. We have applied this technology to carious samples affecting enamel, dentin and deep dentin in order to make a more complete description of the bacterial diversity of dental caries affecting different tissues. In addition, we have performed direct pyrosequencing of the total DNA from the bacterial community at these three stages, allowing a description of the gene functions encoded by the microorganisms present. This metagenomic approach provides the first functional description of the different bacterial populations and their potential role in caries initiation and progression.

Materials and Methods

Sample Collection and DNA Extraction

Supragingival dental plaque and caries samples were obtained from 22 volunteers after signing an informed consent. Sampling procedure was approved by the Ethical Committee for Clinical Research from the DGSP-CSISP (Valencian Health Authority, Spain). Oral health status of each individual was evaluated by a dentist following Nyvad et al. [2003], with nomenclature from the Oral Health Surveys from the WHO. Samples were taken with autoclaved spoon excavators, following Aas et al. [2008]. Plaque material from all tooth surfaces from each individual was pooled. In volunteers with active caries, dental plaque samples were taken without touching cavities. In those cases, material from individual cavities was also extracted and kept separately. Caries samples were classified as enamel caries (including white-spot lesions), dentin caries and deep dentin caries [Aas et al., 2008]. All samples were from active caries, as evaluated by their texture and color (Nyvad et al., 2003). All dentin caries samples were open and deep dentin caries samples selected did not reach the pulp; the absence of pulp exposure was evaluated radiographically, and an absence of apical radiological area was observed for all samples. Volunteers were asked not to brush their teeth for 24 h prior to the sampling. Information was obtained regarding oral hygiene, diet and signs of periodontal disease. Complete information on sample size, teeth sampled and clinical data of donors is included in online supplementary table 2 (for all online suppl. material, see www.karger.com/doi/10.1159/000351663). DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisc., USA), following the manufacturer's instructions, adding a lysozyme treatment (1 mg/ml, 37°C, 30 min). All 22 samples were used for PCR amplification and pyrosequencing of the 16S gene. In addition, 3 representative samples from enamel caries, dentin caries and deep dentin caries were selected for direct, metagenomic pyrosequencing, based on radiographic evaluation and optimal DNA concentration and quality. The sequencing was performed at the Center for Advanced Research for Public Health using the GS FLX sequencer (Roche, Basel, Switzerland) with Titanium chemistry, using $^1/_4$ of a sequenc-

ing plate per sample. Sequences from the 3 metagenomes were deposited at the NCBI Sequence Read Archive under accession number CA_05_4.6: 4447970.3, CA_06_1.6: 4447971.3, CA017_WS: 4450726.3.

PCR Amplification and Pyrosequencing

The first 500 bp of the 16S rRNA genes were amplified in the 22 samples with the universal eubacterial degenerate primers 27F and 533R using the high-fidelity AB-Gene DNA polymerase (Thermo Scientific, Waltham, Mass., USA) with an annealing temperature of 52°C and 20 cycles to minimize PCR biases [Sipos et al., 2007]. The primers were modified to contain the pyrosequencing adaptors A and B and an 8-bp 'barcode' specific to each sample [McKenna et al., 2008]. Barcodes were different from each other in at least 3 nucleotides to avoid errors in sample assignments. Three PCRs were performed per sample, pooling their PCR products before purification, which was done using the NucleoFast PCR purification kit (Macherey-Nagel, Düren, Germany). The final DNA per sample was measured by PicoGreen fluorescence in a Modulus 9200 fluorimeter from Turner Biosystems (Sunnyvale, Calif., USA) and the PCR products mixed in equimolar amounts. PCR products were pyrosequenced from the forward primer end only using a GS FLX sequencer with Titanium chemistry (Roche, Basel, Switzerland) at the Center for Public Health Research (CSISP) in Valencia, Spain. One eighth of a plate was used for each pool of 20 samples.

Sequence Analysis

Reads with an average quality value lower than 20 and/or with more than 4 ambiguities in homopolymeric regions in the first 360 flows were excluded from the analysis. Only reads longer than 200 bp were considered. The ends of the reads, which consistently showed low-quality values, were removed. Sequences were assigned to each sample by the 8-bp barcode and passed through the Ribosomal Database Project classifier [Cole et al., 2009], where each read was assigned a phylum, class, family and genus, as long as the taxonomic assignment was unambiguous within an 80% confidence threshold. To estimate total diversity, sequences were clustered at 97, 95 and 90% nucleotide identity over a 90% sequence alignment length using the RDP pyrosequencing pipeline. For this analysis, sequences over 97% identical were considered to correspond to the same operational taxonomic unit (OTUs), representing a group of reads which presumably belong to the same species [Yarza et al., 2008]. Principal coordinates analysis (PCoA) was performed with UniFrac [Lozupone et al., 2006], with clustering at 97% and the weighed option. This phylogenetic approach takes into account both taxonomically assigned and unassigned reads, using the distance between phylogenetic trees of the 16S gene to construct a matrix for the PCoA. Two-way statistical comparisons in bacterial composition were performed using the UniFrac metric [Lozupone et al., 2006], with 500 permutations and the Bonferroni correction. The DNA sequences were deposited in the MG-RAST server database with access numbers 4486962.3 through 4486985.3, under the project name 'Caries Microbiome'.

For the analysis of the 3 metagenomes, sequences were uploaded to the MG-RAST server [Meyer et al., 2008] and the functional assignment was retrieved at different hierarchical levels, making a table with the counts of reads per functional category and a normalized heat map as explained in Belda-Ferre et al. [2012]. The overrepresentation of functions was estimated by the false discovery rate method applied to metagenomic sequences by the algorithm of White et al. [2009], testing the amount of false-positive predictions (q values) for a given p value of significance. Metagenomic recruitment plots were performed using Nucmer and Promer v3.06, with default parameters [Kurtz et al., 2004] and most abundant bacteria ranked according to the plot coverage [Belda-Ferre et al., 2011].

Results

Bacterial Diversity in Dental Plaque and Carious Lesions

An average of 1,882 16S rRNA high-quality reads were obtained per sample and between 8,000–12,000 per sample type, allowing a fairly complete description of bacterial diversity in dental plaque samples of unaffected teeth and in carious lesions affecting different tissues. When the number of 16S rRNA reads analyzed is plotted against the number of species-level phylotypes, a rarefaction curve is obtained in which the sampling effort is related to bacterial diversity. These curves show a dramatic decrease in bacterial diversity of all caries samples relative to the intact enamel surfaces from individuals with caries (fig. 1). Specifically, dental plaque samples from intact enamel reached 1,015 species-level OTUs (an approximation of the number of species based on the 16S rRNA sequences), whereas white-spot lesions affecting enamel had only 193 OTUs. These lesions showed the lowest level of diversity of caries samples, with dentin and deep dentin caries samples attaining 350 and 290 OTUs. The higher microbial diversity of dental plaque suggests that bacteria inhabiting carious environments are a small subset of the bacterial community from intact enamel and that cavities represent selective niches which only specialized bacteria are able to colonize and exploit. The fact that the lowest level of diversity is found in enamel lesions indicates that this is the most stringent niche in human teeth, suggesting that the acidic environment probably acts as the main selective force reducing the number of species able to thrive in enamel caries.

Bacterial Composition

Taxonomic assignment of the 16S rRNA reads indicated that the bacterial composition varied depending on the tissue affected. Dental plaque samples showed the highest number of assigned genera, which appeared to be more equally distributed than in carious samples, where some genera disappeared and others became more dominant (fig. 2a). In dentin caries, *Streptococcus* and *Prevotella* increased in proportion whereas *Neisseria*, *Cap-*

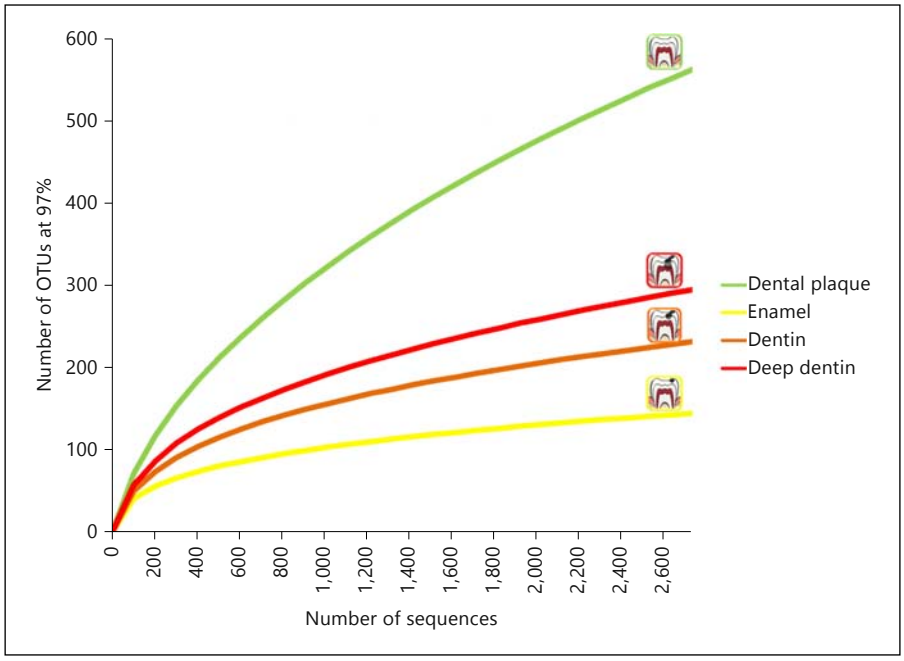

Fig. 1. Bacterial diversity in dental plaque and caries lesions. The rarefaction curves shown indicate the relationship between sequencing effort and the estimated number of species in dental plaque samples, enamel caries, dentin caries and deep dentin caries lesions. The number of species-level phylotypes was calculated by pooling samples from the same sample type and clustering the sequences at 97% of sequence identity. This number of OTUs is an estimate of species-level phylotypes. Carious lesions are between 2 and 5 times less diverse than dental plaque samples from sound tooth surfaces.

nocytophaga and *Fusobacterium* significantly decreased in relative numbers. Interestingly, *Lactobacillus* species, which have been associated with dental caries because of their acidogenic potential, only appeared in deep dentin caries. This is in agreement with other reports indicating that *Lactobacillus* species do not play a central role in caries initiation but in progression of the lesion [Shah et al., 2011]. *Bifidobacterium* was detected at very low levels but although this could partly be due to the low amplification efficiency of this genus with universal primers [Hill et al., 2010], metagenomic recruitment data also indicated a small contribution of this bacterium to the caries community. The increase in streptococcal species during caries progression was not due to mutans streptococci, which in fact appeared to diminish in dentin caries, as shown by the complete absence of recruitment of the *Streptococcus mutans* genome in dentin and deep dentin cavities (online suppl. fig. S1). However, some proteolytic bacteria like *Prevotella*, which increase in proportion in dentin cavities, have been shown to be saccharolytic and tolerant to the acidic environment [Takahashi et al., 1997] and could also be acidogenic [Aamdal-Scheie et al., 1996]. Although the assignment of streptococcal species using partial 16S gene sequences has to be taken with care, *Streptococcus mitis* appeared to be the dominant member of this genus in dentin cavities (fig. 2b). Strains most similar to *Streptococcus cristatus* appeared to increase in deep dentin cavities whereas *Streptococcus gordonii* and *S. mutans* increased in enamel caries compared to dental plaque from sound tooth surfaces (fig. 2b). Thus, the role of multiple organisms other than mutans streptococci for caries initiation and progression should be further studied. The metagenomic sequences obtained by direct pyrosequencing of total DNA allowed the search for fungal and protozoan genes. Genes from different *Candida* species (with best hits to *Candida albicans*, *Candida dubliniensis* and *Candida tropicalis*) were found in enamel and dentin lesions; given that this yeast has been related to an increase in caries incidence [Raja et al., 2010], its presence at different stages of caries indicates that they may play a role in the disease. Although the relative presence of yeasts is very low when compared to the bacterial component of the community, these organisms are more resistant to standard cell lysis procedures than bacteria and therefore future work specifically aimed at studying fungal presence and diversity in caries lesions is required.

To investigate the similarity in bacterial composition among individual samples, a PCoA was performed. Although there was some degree of overlap between the different sample types, a clear pattern emerged in which caries lesions tended to cluster together with samples of the same kind (fig. 3). The bacterial composition of enamel caries was significantly different from those of dental plaque and dentin caries (UniFrac distance, $p < 0.002$ in both cases). The presence of a distinctive, tissue-depen-

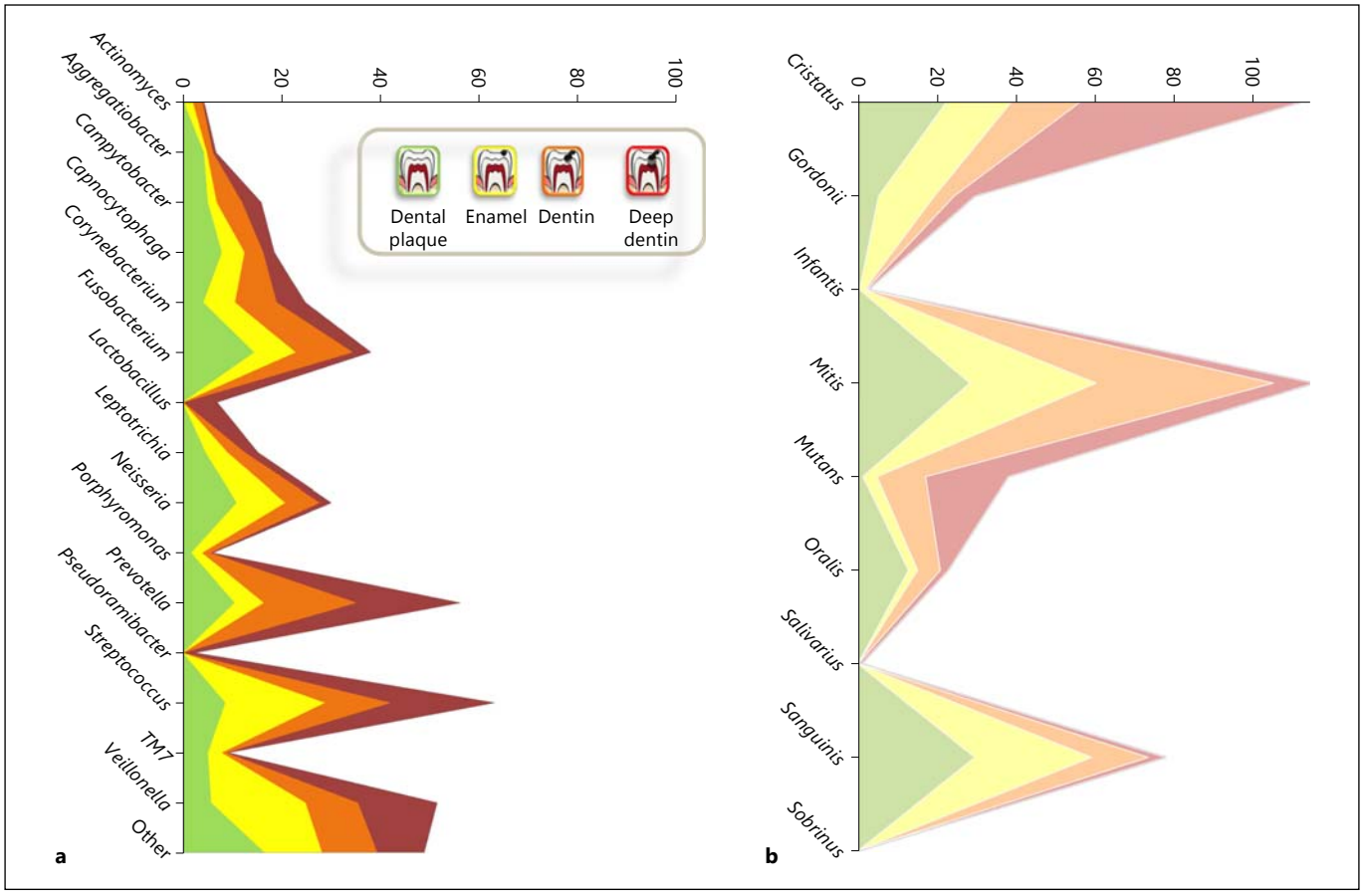

Fig. 2. Bacterial composition in dental plaque and carious lesions. Graphs indicate the taxonomic assignment at the genus level of PCR-amplified 16S rRNA pyrosequences in dental plaque samples, enamel caries, dentin caries and deep dentin caries samples (**a**) and the taxonomic assignment at the species level for streptococci (**b**). Several genera and *Streptococcus* species increase and others decrease in proportion as the disease progresses.

dent microbiota suggests that the etiological factors of caries can be different at different stages of caries progression, as it is confirmed by the data on gene function representation.

Functional Profile of Caries Progression

The second approach employed was a metagenomic study of the bacterial communities from white-spot, dentin and deep dentin lesions. We aimed to test the hypothesis that the functional profile of enamel and dentin caries communities is different. Functional assignment of 547,102 sequences totaling 171.87 Mbp from the total bacterial DNA against protein databases produced a functional profile which could be compared to that obtained by the same approach from dental plaque samples of individuals with and without caries [Belda-Ferre et al., 2012]. Using a clustering algorithm that groups samples

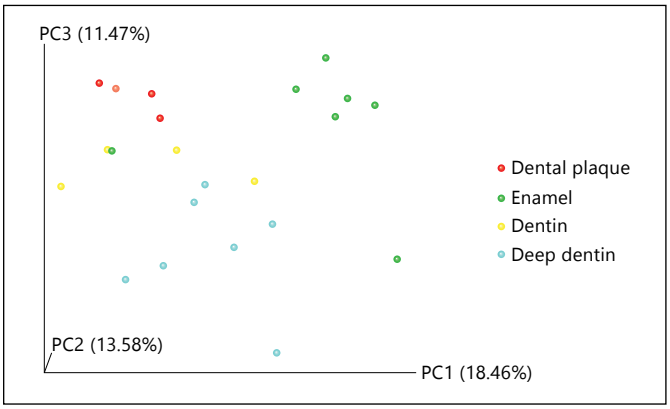

Fig. 3. Variability in bacterial composition among samples. The PCoA plot separates samples according to caries status. Red dots represent dental plaque samples from sound tooth surfaces; green dots correspond to enamel caries lesions, yellow dots to dentin cavities and blue to deep dentin cavities.

according to the proportion of gene functions encoded in their DNA (dental plaque samples from individuals who had never suffered from dental caries clustered together) indicated that bacteria inhabiting the healthy teeth have a distinct set of genes. Dental plaque collected from healthy teeth in individuals with caries at the moment of sampling had a functional profile most similar to that of white-spot lesions, whereas dentin lesions had a different profile from the rest (fig. 4). Thus, bacteria of caries samples affecting different tissues had a different functional profile.

Looking into detail at those functions overrepresented in each sample type revealed the main features of the ecosystem and the microbial contribution to dental caries. As expected, enamel caries samples showed an overrepresentation of genes encoding complex carbohydrate fermentation, adhesion to cell surfaces and acid stress responses (q and p values of overrepresented functions in enamel vs. dentin metagenomes is shown in online suppl. table 1). However, genes for dietary sugar fermentation and pH stress were at very low levels in dentin lesions, which showed instead an overrepresentation of genes for the metabolism of monosaccharides ($q = 1.95 \times 10^{-9}$, $p = 3.98 \times 10^{-11}$) and disaccharides ($q = 3.58 \times 10^{-21}$, $p = 2.43 \times 10^{-23}$), osmotic stress such as glycerol uptake proteins and aquaporins, adhesion to collagen and fibronectin, and a wide arsenal of peptidases, including collagenases and aminopeptidases. There is also an overrepresentation of enzymes which degrade human glycans and transport their sugar components like mannose (including mannose 6-phosphate isomerase, phosphomannomutase, and mannoside ABC transporters) and sialic acid (including a sialic acid transporter and an N-acetyl neuraminate synthase), and galactose (including galactokinase and α- and β-galactosidase), although the latter is also a dietary carbohydrate contained in lactate. In deep dentin samples, genes related to the immune response of the host were overrepresented. These included the sortase *srt*A, which has been shown to have an antiopsonization effect and be vital for bacterial colonization and infection in *Streptococcus sanguinis* [Yamaguchi et al., 2006], and genes for allantoin utilization. Allantoin stimulates cellular proliferation and helps to substitute necrotic for new tissues and must be at high levels at the pulp vascular tissues, exemplifying the degree to which deep dentin bacteria have specialized in using nutrient sources that are available at that specific niche where ingested food may not reach, especially in hidden cavities. Iron-dependent siderophores are also overrepresented in these samples, suggesting that iron must be a very scarce mineral within dentin.

Discussion

A Microbial-Succession, Tissue-Dependent Hypothesis of Dental Caries

When we integrate the available information on caries microbiology with our own metagenomic data on caries lesions, dental caries emerges as a two-step process in which there is a succession of microorganisms. The complex microbial composition of cavities at different stages is clearly not consistent with the specific plaque hypothesis and supports a polymicrobial origin. S. *mutans* increases in proportion in caries lesions relative to dental plaque samples, but appears to be at very low frequencies, as previously reported [Gross et al., 2012]. The microbial community undergoes a dramatic reduction in the number of species from a healthy to a diseased site (fig. 1). By considering the bacterial community within cavities, and looking at the predicted output of their activity, we have gained insights into the different ecosystems the carious process goes through. The functions those organisms encode support a view in which enamel caries lesions are dominated by acidogenic organisms which ferment dietary carbohydrates and where mutans streptococci are relatively common. This environment is acidic and enamel gets demineralized. However, when a channel is demineralized by their action and dentin is reached, the community changes significantly and is predominantly proteolytic, and the main environmental pressure has an osmotic nature. At this stage, the tissue has a mineralized component with a lower proportion of hydroxyapatite and a higher critical pH which was estimated to be as high as 6.7 [Hoppenbrouwers et al., 1987], although lower values in the range 5.22–5.66 have been reported by recent in vitro work in root surfaces of human teeth [Shellis, 2010]. In addition, pH values in dentin caries lesions appear to be variable [Hojo et al., 1994]. Thus, the low frequency of acid stress and carbohydrate fermentation genes reported here could be influenced by the limited sample size of the study and should be confirmed in larger data sets. Although attempts to culture dentin bacteria with collagenolytic activity have not been successful, our metagenomic data show that collagenases are overrepresented, especially in *Prevotella* species. The data suggest that dental caries cannot be considered a classical infectious disease [Fejerskov, 2004] in the sense that Koch's postulates are not applicable, the microbial players involved change through time depending on the tissue affected, and multiple causes are responsible for the progressing lesion at different stages of the caries process.

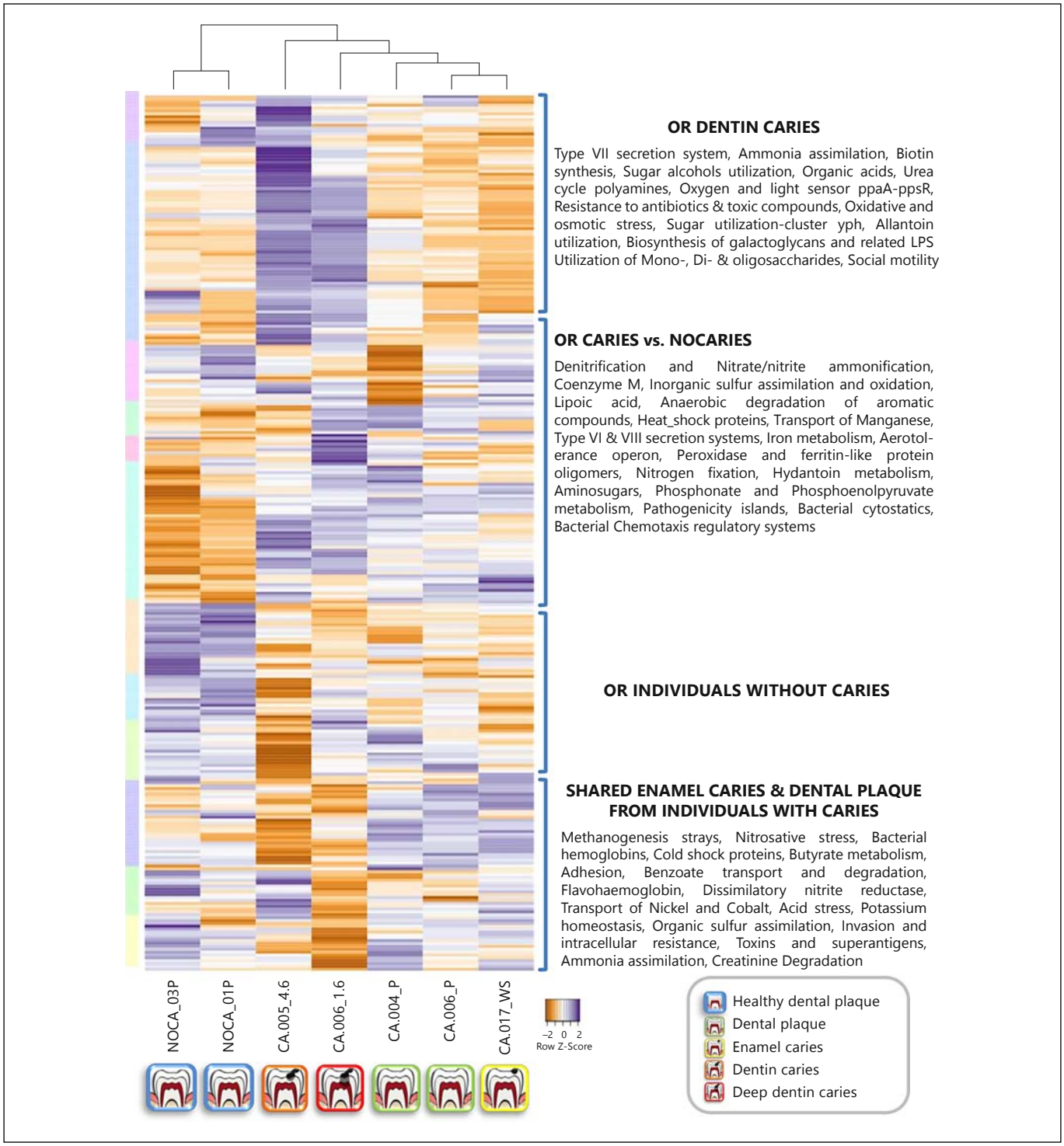

Fig. 4. Functional profile of the microbiota present in dental plaque and cavities at different stages of caries progression. Each line represents a functional category, as assigned by the SEED system [Meyer et al., 2008], depicted with a color indicating whether each function is over- or underrepresented in the different samples. Each column corresponds to the data from individual samples of dental plaque from donors who never suffered from dental caries (teeth surrounded by a blue square), dental plaque of healthy tooth surfaces from donors with more than 10 cavities at the moment of sampling (teeth surrounded by a green square), enamel caries (white-spot lesions), dentin caries and deep dentin caries lesions. Statistically significant differences in relevant functions between enamel and dentin caries samples are indicated.

Clinical Relevance

There are several clinical observations that can be explained in the light of the microbial hypothesis of dental caries. One of them is the funnel shape of enamel lesions. Tooth radiographs consistently show that the aperture on the external side of the enamel lesion is wider than that on the dentin side [Ekstrand et al., 1991]. If the internal pH in dentin lesions was also acidic, a double-funnel shape would be expected. However, radiographs of carious teeth rarely show a reduction of enamel width from the inside, indicating that the global output of the bacterial community in dentin caries lesions, although it contains both acidogenic and proteolytic organisms, may indeed be considerably less acidic than the enamel caries microbiota [Zaura et al., 2002]. Compared to enamel lesions, dentin cavities – particularly hidden or unexposed lesions – are expected to have lower availability of fermentable dietary carbohydrates and this could influence the acidogenicity of the environment. Nevertheless, due to the chemical properties of dentin hydroxyapatite, critical pH is higher than that of enamel [Hoppenbrouwers et al., 1987] and tissue demineralization can proceed even in moderately acidic conditions. The consequences of proteolytic metabolism in dentin microorganisms should also be evaluated, as the by-products of proteolysis could in fact contribute to an increase in the pH. Proteolytic microbial activity in dentin has also been shown to induce production of human metalloproteases, which can further degrade the tissue [van Strijp et al., 2003; Chaussain-Miller et al., 2006].

The progression of cavities is best seen in interproximal lesions because of the absence of occlusal forces and the limited availability of food remnants. In these circumstances, the dentin lesion expands radially and reaches the inside part of enamel without degrading it, again suggesting a limited acidity unable to demineralize enamel. In coronal cavities, however, masticatory forces can break the enamel located over the dentin cavity, giving the wrong impression that the enamel has been degraded by the caries when in fact it has been fractured by mastication. In the absence of enamel breakdown by mastication, however, the caries mineral dissolution merely forms a funnel-shaped channel through enamel to an entirely new niche with stringent anaerobic and osmotic characteristics, under different immunological challenges and where nutrient availability from food is limited. Physiological, morphological and genetic studies of bacterial isolates from dentin show some of their immune evasion mechanisms [Jontell et al., 1998], highlight an important ability to liberate sugars from human glycoproteins [Paddick et al., 2005] and a capacity to adhere to dentinal tissue [Love et al., 1997], and show that the size and shape of these bacteria allow them to proceed through the dentinal channels [Love and Jenkinson, 2002]. Our metagenomic data also illustrate this formidable adaptation to survive in the dentinal tissue and anticipate fascinating mechanisms of nutrient utilization and interactions with the immune system.

One of the pulp tissue's responses against bacterial invasion is the formation of reactive dentin. In this altered tissue the dentinal channels are irregularly arranged and blocked by the formation of crystals, preventing the advancement of bacteria towards the pulp [Love and Jenkinson, 2002]. In addition, the tissue is devoid of water and proteins, including collagen [Nanci, 2012]. If acidity was the only cause of caries progression, reactive dentine should be degraded by sugar-fermenting organisms, but this modified tissue in fact reduces effectively the progression of the lesion. In the light of the microbial hypothesis presented here, dietary sugar-fermenting organisms are a minority in dentin caries and do not have access to fermentable complex carbohydrates, whereas dentin dwellers are also specialized in proteolytic digestion of dentinal tissue and would therefore not be able to create the acidogenic environment that presumably would be necessary to break reactive dentin.

Our data underline that a better accuracy in caries description would be desirable, as individuals with the same oral health indexes could vary considerably in the presence of cavities at different stages. An example is given by *Lactobacillus*, which we have found only representative in deep dentin caries. Thus, we believe that even if saliva sampling is found to be appropriate for epidemiological studies, etiological research must focus on individual lesions.

If caries disease is a tissue-dependent process, standard preventive measures such as dietary reduction of fermentable sugars [Marsh, 2003], pH regulation strategies [Stookey, 2008] and targeting of acidogenic species for immunization [Abiko, 2000] would still represent valid approaches because they would be centered in reducing the initiation of cavities. However, caries progression would involve a different set of bacterial players and tissue-degrading processes and is likely to be relatively independent of diet. Thus, buffering of acidity and diet control may have a reduced effect once the lesion has demineralized enamel and entered dentinal tissue. In addition, active and passive immunization strategies should not be aimed exclusively at one species, but should use antigens which can target different bacteria involved in the caries

process [Mira et al., 2004; Mira, 2007]. Even within enamel caries, the diversity of bacteria is wide (fig. 1) and includes many potentially acidogenic bacteria outside mutans streptococci (fig. 2). Thus, our data do not support the specific plaque hypothesis [Loesche et al., 1975; Loesche, 1992], as it suggests that caries initiation and progression appear to be the result of microbial consortia. Further work should aim to determine whether those bacterial consortia are formed by specific bacteria or whether the acidogenic output responsible for enamel caries initiation can be produced by shifts in the bacterial community equilibrium regardless of their specific composition, as the ecological hypothesis has proposed [Marsh, 1991; Takahashi and Nyvad, 2011].

We hope that the current study stimulates further metagenomic work on caries lesions, especially hidden cavities where contamination from nondentin bacteria would not exist. Culturing the etiological agents of dentin caries could also prove fruitful as their proposed nonacidogenity and proteolytic and glycanolytic capabilities could be experimentally tested. In order to test cavity acidity, accurate in vivo pH measurements of dentine lesions, especially nonexposed lesions, are required, and recent advances in pH microscopic probes and pH-dependent dyes [Hiraishi et al., 2003; Schlafer et al., 2011] could make this possible in the near future. In addition, it has to be kept in mind that DNA-based metagenomic approaches cannot distinguish live from quiescent or dead bacteria and future studies should focus on RNA-based or protein-based methodologies (metatranscriptomics and metaproteomics approaches, respectively) to focus on the role played by the active fraction of oral microbiota [Nyvad et al., 2013].

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Disclosure Statement

There are no potential conflicts of interest for any of the authors.

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Journal of Oral Microbiology

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REVIEW ARTICLE

Metatranscriptomics reveals overall active bacterial composition in caries lesions

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Background: Identifying the microbial species in caries lesions is instrumental to determine the etiology of dental caries. However, a significant proportion of bacteria in carious lesions have not been cultured, and the use of molecular methods has been limited to DNA-based approaches, which detect both active and inactive or dead microorganisms.
Objective: To identify the RNA-based, metabolically active bacterial composition of caries lesions at different stages of disease progression in order to provide a list of potential etiological agents of tooth decay.
Design: Non-cavitated enamel caries lesions ($n = 15$) and dentin caries lesions samples ($n = 12$) were collected from 13 individuals. RNA was extracted and cDNA was constructed, which was used to amplify the 16S rRNA gene. The resulting 780 bp polymerase chain reaction products were pyrosequenced using Titanium-plus chemistry, and the sequences obtained were used to determine the bacterial composition.
Results: A mean of 4,900 sequences of the 16S rRNA gene with an average read length of 661 bp was obtained per sample, giving a comprehensive view of the active bacterial communities in caries lesions. Estimates of bacterial diversity indicate that the microbiota of cavities is highly complex, each sample containing between 70 and 400 metabolically active species. The composition of these bacterial consortia varied among individuals and between caries lesions of the same individuals. In addition, enamel and dentin lesions had a different bacterial makeup. Lactobacilli were found almost exclusively in dentin cavities. Streptococci accounted for 40% of the total active community in enamel caries, and 20% in dentin caries. However, *Streptococcus mutans* represented only 0.02–0.73% of the total bacterial community.
Conclusions: The data indicate that the etiology of dental caries is tissue dependent and that the disease has a clear polymicrobial origin. The low proportion of mutans streptococci detected confirms that they are a minority and questions its importance as the main etiological agent of tooth decay. Future experimental work should be performed to confirm the cariogenicity of the identified bacteria.

Keywords: *16S rRNA*; *pyrosequencing*; Streptococcus mutans; *polymicrobial disease*; *tissue-dependent hypothesis*; *caries etiology*

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It has been estimated that approximately 50% of oral bacteria have not been cultured to date (1). Classical studies based on microbial culture established mutans streptococci and lactobacilli as the main causative agents of dental caries (2, 3). However, other microbial species were also isolated from caries lesions and have been related to the disease, including bifidobacteria and *Scardovia* (4, 5). Furthermore, the application of molecular cloning and Sanger sequencing to study carious lesions at different stages of the disease revealed that although *Streptococcus mutans* levels correlated with disease severity, it could not be always amplified by polymerase chain reaction (PCR) whereas other bacteria were present, including *Prevotella*, *Atopobium*, and *Propionibacterium* (6). When the metagenomic DNA from individual dentin caries samples was directly sequenced without the need for PCR, the genus *Veillonella* appeared dominant within a surprisingly diverse community (7), underlining the varying nature of microbial composition in cavities. The application of pyrosequencing to PCR products of the 16S rDNA gene has become an extremely powerful approach, revealing that cavities are extraordinarily diverse ecosystems (8)

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where *S. mutans* accounts at most for 1.6% of the carious lesion bacterial community (9).

A drawback of these DNA-based studies is that the PCR step may amplify DNA from inactive or even dead microorganisms, making it necessary to determine the functional bacteria that effectively contribute to the disease (10). A way to achieve this is to perform the 16S gene amplification starting from RNA material, given that the amount of rRNA material in bacterial cells is known to be related to their degree of metabolic activity (11, 12). In the current work, we have performed PCR amplification of RNA extracted from enamel and dentin caries lesions, after a reverse-transcription step (13). The obtained PCR products were then pyrosequenced with the aim of characterizing the active bacterial composition of cavities.

Materials and methods

Sample collection

All donors signed a written informed consent and the sampling procedure was approved by the Ethics Committee from the DGSP-CSISP (Valencian Health Authority), with reference 10/11/2009. All donors attended the University of Santiago Dental Clinic, had not been treated with antibiotics or antifungals in the previous 6 months, had all 28 teeth present (excluding third molars) and had not suffered from any systemic disease. Clinical data are shown in Supplementary Table 1. All caries lesions sampled were active lesions as assessed by their texture and colour, and active white spot lesions were identified because they appear chalky white, opaque, and rough (14). All enamel caries collected ($n=15$) were non-cavitated ('white spot' lesions) and were collected with sterile spoon excavators. Supragingival dental plaque samples in caries-bearing individuals were taken 24 hours after tooth-brushing from vestibular and palatine surfaces of the teeth using the same procedure (15). Teeth were not dried before sampling. Unstimulated saliva samples were collected by drooling, as previously described (15). Open dentin caries lesions ($n=6$) were sampled directly with a sterile spoon excavator, after removing the top, biofilm layer in contact with the oral environment. All carious teeth were isolated with rubber dam to reduce the risk of saliva contamination. Unexposed dentin cavities ('hidden dentin lesions', $n=6$) were assessed radiographically, and a water-cooled diamond bur in an air-turbine handpiece was used to drill the enamel. Water circulation was stopped right before reaching dentine tissue to minimize contamination, and the dentine lesion was hand excavated with a sterile spoon excavator. Radiographic images revealed no evidence of pulp necrosis in any dentin carious lesion sampled. Samples were eluted in 500 µl of PBS buffer and immediately frozen at -80°C. RNA concentrations obtained ranged from 12 to 120 µg/µl. All samples were diluted to 12 µg/µl before PCR was performed.

RNA extraction, PCR amplification and sequencing

RNA was extracted by a combination of physical and chemical lysis. Carious lesions samples were suspended in 500 µl sterile saline solution containing 0.1 and 0.5 mm glass beads, and subject to 50 Hz beating for 2 min in a Tissuelyzer II (QIAGEN, Limburg, The Netherlands) followed by 5 min on ice three times. Chemical lysis, RNA extraction, and DNAse treatment were then performed using the RNA/DNA Masterpure Extraction kit (Epicentre, Hamburg, Germany) following the manufacturer instructions with the addition of a lysozyme treatment at 37°C for 30 min.

Single-stranded cDNA was constructed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Grand Island, NY) in 20 µl reactions, with several modifications, following Benítez-Páez et al. (13). Universal bacterial primers 8F (5′-TCAGAGTTTGATCMT GGCTCAG-3′) and 788R (5′-GGCCVGGGTATCTAA TCC-3′) were used to partly amplify the 16S rRNA gene from the single-stranded cDNA in two 50 µl reactions, following the PCR and purification conditions described by Simón-Soro et al. (15). In three cases, there was enough carious material to obtain both DNA and RNA. In those three individuals, DNA was also extracted from dental plaque and drooling saliva following reference (15) and used for comparison. Purified PCR products were measured in a Modulus fluorimeter (Turner Biosystems, Madison, WI) and mixed in equimolar amounts in two pools of 14 samples, which were sequenced in 1/8 of a plate in the GS-FLX pyrosequencer (Roche, Basel, Switzerland) with Titanium-plus chemistry.

Sequence analysis

To increase accuracy in taxonomic assignment, only reads longer than 400 bp were selected. Chimeric PCR products were filtered out using the software Uchime, (16) and the reads were end-trimmed and quality filtered following Cabrera-Rubio et al. (17). Sequences were separated by the sample-specific 8-bp barcode and assigned to a genus using the Ribosomal Database Project classifier, with an 80% confidence threshold (18). Sequences were clustered at 97% nucleotide identity over 90% sequence alignment length, and rarefaction curves were obtained using the RDP pyrosequencing pipeline. An attempt was made to assign the reads to the species taxonomic level. In order to do this, a curated database was constructed with the full-length 16S sequences of all species present in the Ribosomal Database Project that belonged to the genera *Streptococcus*, *Lactobacillus*, and *Veillonella*. A BlastN (19) was then performed against this database with the reads that had been previously assigned to the above-mentioned genera and that were >500 bp. The top hit from each sequence comparison was selected if the alignment length was >500 bp, and the sequence identity $>99\%$.

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Results and discussion

After end-trimming and quality filtering, a total of 132,599 sequences were obtained for 15 enamel lesions and 12 dentin lesions. Chimeras reached 1.51% of the total and were filtered out. An average of 4,911 reads were obtained per sample, with a mean length of 661 bp.

Reads were clustered at 97% sequence identity, which is the consensus threshold for bacterial species boundaries (20). These Operational Taxonomic Units (OTUs) can therefore be used to estimate the number of species in a sample. Enamel caries lesions were the least diverse, with a median of 177.7 bacterial species, whereas the estimates for open and hidden dentin cavities were 250.7 and 201.2, respectively (Fig. 1). Bacterial diversity levels varied not only between individuals but also between caries samples from the same individual, as also shown by rarefaction curves where the sequencing effort is plotted against the estimated number of species (Supplementary Fig. 1). The data suggest that white spot lesions appear to be a very restrictive niche, whereas open dentin cavities are the most diverse, even though only the inner layer of the lesion was selected for RNA extraction. Hidden dentin lesions were less diverse than deep dentin lesions from open dentin cavities, suggesting that the latter have a supply or microorganisms from the oral cavity. Open dentin lesions displayed also the highest variability, in accordance to their exposure to the salivary environment. The existence of such a high level of diversity even in the active fraction of the bacterial community confirms that the high number of organisms detected in caries lesions is not due to dead or inactive species and that dental caries is a polymicrobial disease, where multispecies microbial consortia are metabolically active in the lesions.

Active bacterial composition was significantly different between enamel and dentin cavities (Unifrac distance $p < 0.001$), suggesting that these microbial communities are tissue dependent. Streptococci, *Rothia*, *Leptotrichia,* and *Veillonella*, for instance, were at higher levels in enamel carious lesions, whereas *Lactobacillus*, *Shlegelella*, *Pseudoramibacter*, and *Atopobium* appeared to be clearly associated with dentin lesions (Fig. 2, top). There is also a high number of minority species (found at $<1\%$ proportion, indicated as 'Other' in Fig. 2) that were exclusively found in enamel lesions and a few of them were only found in dentin carious lesions. The latter included *Tannerella*, *Olsenella*, *Filifactor*, and *Treponema*. Given

Fig. 1. Active bacterial diversity in caries lesions. Data show the estimated number of bacterial species (Operational Taxonomic Units, or OTUs), as calculated by the Chao Richness Index on the sequences clustered at 97% nucleotide identity. Patients' codes are displayed on the right panel and samples from the same individual are depicted with the same symbol. Data are shown for non-cavitated enamel caries (left), open dentin caries (middle), and unexposed, hidden caries (right). Medians from each group are shown for reference. Note that several caries samples are available for a few individuals.

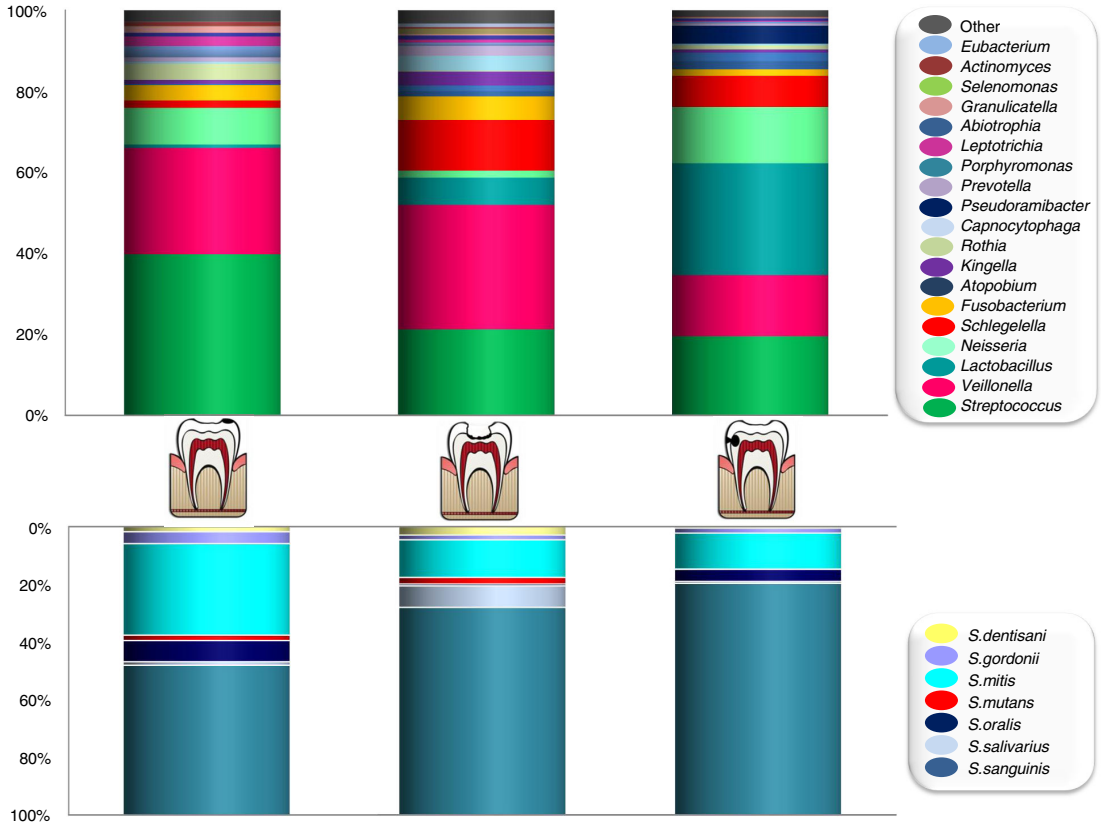

Fig. 2. Taxonomic composition of active bacteria in caries samples as determined by pyrosequencing of the 16S rRNA gene. Graphs show the proportion of bacterial genera found at >1% of the total (top panel) and the proportion of different streptococcal species (lower panel), calculated as the means of all carious samples ($n=15$ for enamel lesions, $n=6$ for open dentin lesions, and $n=6$ for closed dentin lesions). Data are shown for non-cavitated enamel caries (left), open dentin caries (middle), and unexposed, hidden caries (right).

the high frequency of streptococci, an effort was made to identify streptococcal sequences at the species level (Fig. 2, lower panel). *Streptococcus sanguinis* increased significantly in dentin cavities, whereas *S. mitis* was more abundant in enamel lesions. In relation to *S. mutans*, which is probably the most studied caries-associated species, a dramatically low proportion was found in all samples, ranging from 0.73% in enamel lesions to 0.48% in open dentin and 0.02% in hidden dentin lesions. The low proportion detected confirms that this species is a minority (8, 9) and questions its importance as the main etiological agent of tooth decay (21). Also, bacterial counts of lactobacilli frequently used to predict caries risk in diagnostic tests may not be informative given that they are virtually absent in enamel lesions, and this would imply that they are probably not involved in caries initiation.

There were three cases in which DNA from the same caries lesions, together with saliva and supragingival dental plaque DNA from sound teeth surfaces, could also be obtained. These pilot data show that when the DNA-based bacterial composition of a lesion is compared to the RNA-based composition of the same individual lesion, a clear difference was observed (Fig. 3, inner circles). The former, metagenomic approach, shows the total bacterial community, whereas the metatranscriptomic approach describes the active players in that community and we propose the latter procedure is therefore a closer approximation to the disease etiology. Other samples taken from the oral cavity of the same individual, like supragingival dental plaque or unstimulated saliva, show a different bacterial composition (Fig. 3, outer circles), where the detection of cariogenic bacteria is hampered by the predominance of microorganisms from other buccal niches (15). For instance, *Megasphaera* is found in saliva but passed virtually undetected in caries lesions, and *Porphyromonas* was found at considerable proportions in saliva and supragingival dental plaque, but is a minority in enamel lesions.

Conclusion

The number of active species in lesions found in the current work is lower than in previous studies based on ribosomal DNA (from 206 in white spot lesions and 379 in

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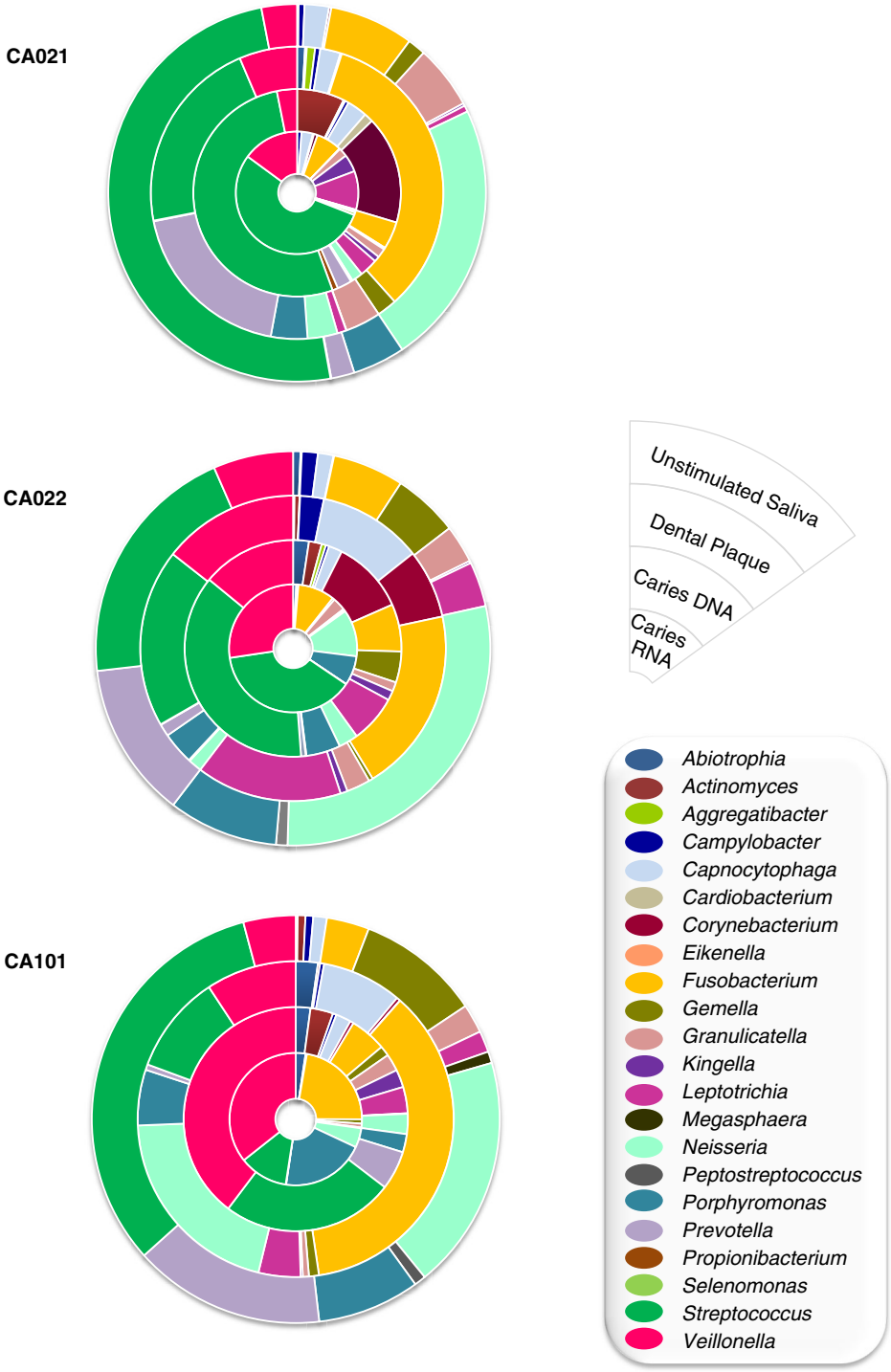

Fig. 3. Bacterial composition in different oral samples from three caries-bearing individuals. Data show the proportion of bacterial genera for unstimulated saliva (DNA-based), supragingival dental plaque of sound teeth surfaces (DNA-based), enamel caries lesions (DNA-based) and enamel caries lesions (RNA-based), from the outer to the inner circles.

dentin lesions (9) to 177 and 201 [this study], respectively). This list can be taken as a first approximation of the repertoire of microorganisms potentially involved in caries initiation and progression, as the taxonomic description of caries lesions is a vital step in determining the etiology of the disease. Several taxonomic groups are nevertheless likely to be under-represented, such as G + C rich bacteria including *Actinomyces* and *Bifidobacterium*, which are known to be poorly amplified by 'universal' primers (22). Mutans streptococci, however, are readily amplified with

the primers used in this and other studies (21), and their low proportion is probably not a PCR artifact, as also proposed by other authors in DNA-based studies (8, 9). It has also to be borne in mind that fungal species have also been detected in caries lesions, and proposed to contribute to cariogenicity (23). Thus, the use of bacterial primers cannot detect fungal organisms, and it would be necessary to perform high-throughput sequencing analyses of fungal species to gain insights into the diversity and contribution of these microorganisms to dental caries. Nevertheless, the varying polymicrobial nature of cavities shown in the current manuscript and the currently accepted ecology-based hypothesis of caries disease (24, 25) underline that the functional output of the microbial community is probably more important than its species-composition in order to understand and combat the disease. Thus, in the future, the application of high-throughput direct sequencing to the RNA extracted from oral samples (13) will provide an opportunity to identify not only the active microbial composition but also the expressed genetic repertoire underlying disease initiation and progression. From an applied point of view, the polymicrobial etiology of dental caries underlines that diagnostic and therapeutic strategies directed at single species are likely to be unsuccessful.

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Trends in Microbiology

Microbiology of dental caries

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Solving the etiology of dental caries

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For decades, the sugar-fermenting, acidogenic species *Streptococcus mutans* has been considered the main causative agent of dental caries and most diagnostic and therapeutic strategies have been targeted toward this microorganism. However, recent DNA- and RNA-based studies from carious lesions have uncovered an extraordinarily diverse ecosystem where *S. mutans* accounts only a tiny fraction of the bacterial community. This supports the concept that consortia formed by multiple microorganisms act collectively, probably synergistically, to initiate and expand the cavity. Thus, antimicrobial therapies are not expected to be effective in the treatment of caries and other polymicrobial diseases that do not follow classical Koch's postulates.

Microbiology of dental caries

Classical Koch's postulates contend that a specific microorganism can be found to be responsible for an infectious disease when it invades a host, a principle that has been assumed to be correct for most microbial infections. Although the identification of asymptomatic carriers readily showed that the postulates have important limitations and the original formulation has been modified with the introduction of genetic techniques [1], the principles proposed by Koch have remained a cornerstone in microbiology. Probably due to this, when the sugar-fermenting, acidogenic species *S. mutans* was isolated in the 1920s from carious lesions, it was considered to be the etiological agent of dental caries [2]. Dental caries is considered the most prevalent human disease, affecting 80–90% of the world population [3]. In children, it appears to have a fivefold higher prevalence than asthma, which is the second most prevalent disease. For decades, mutans streptococci have been considered the main causative agent of the disease [4] and most diagnostic, preventive, and therapeutic strategies have been targeted toward this microorganism (see, for example, [5–7]). However, other microbial species were also isolated from carious lesions and have been related to the process of tooth decay, including lactobacilli [8] and bifidobacteria [9]. The introduction of molecular approaches to study the human microbiome revealed that the oral ecosystem is inhabited by hundreds of bacterial species [10], most of which are considered commensals, and that species regarded as pathogens are frequently found in healthy individuals, although at lower levels than in diseased subjects [11]. An important hurdle in determining the etiology of tooth decay is that many samples were not taken from the disease site itself but from other, noninvasive samples such as saliva, which does not represent the cariogenic microbiota (Box 1). However, in a seminal work, Aas and collaborators obtained over 1200 clones of the 16S rRNA gene from dental plaques and carious lesions at different stages of the disease [12]. This work showed that *S. mutans* could not be PCR amplified in a significant proportion of samples and other bacteria such as *Atopobium*, *Prevotella*, and *Propionibacterium* appeared to be associated with the disease. Recent work added *Scardovia wiggsiae* as a new etiological agent of severe early childhood caries [13]. In recent years, the use of second-generation sequencing and metagenomic techniques has uncovered an extraordinarily diverse ecosystem where *S. mutans* accounts only for 0.1% of the bacterial community in dental plaque and 0.7–1.6% in carious lesions [14,15]. When the DNA of samples from dentin caries was directly sequenced, obviating cloning or PCR techniques, *Veillonella* appeared as the most common genus [16], underlining the varying nature of microbial composition in cavities. However, these DNA-based studies may quantify dead, transient, or inactive microorganisms that do not contribute to the disease, inflating estimates of diversity and introducing noise in the analysis [17]. Thus, the application of high-throughput sequencing to the RNA extracted from oral samples finally provides an opportunity to identify the metatranscriptome; that is, the active microbial composition and expressed genetic repertoire underlying disease initiation and progression.

The first of these RNA-based studies on the surface of teeth [18] studied the active microbial communities in oral biofilms before and after a meal, identifying the bacteria that increase their activity after food ingestion, with the premise that these organisms may be involved in sugar fermentation and acid formation. Metatranscriptomic data indicate that the active microbiota is a subset of the total microbial composition in oral biofilms [19] but is still extraordinarily diverse. In addition, the RNA-based estimates of diversity indicate that different microbial consortia are formed in the dental plaque of different individuals. Thus, determining the active microbiota in carious lesions may finally unravel the elusive etiology of the disease, paving the way for diagnostic and preventive tools.

The active microbiota of cavities

The first RNA-based estimates of bacterial diversity in cavities are shown in Figure 1, putatively representing the microbial consortia that are actively contributing to the disease. This approach shows an average of eight active

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genera at a presence of over 1% in both enamel and dentin lesions. However, each lesion appears to harbor a different combination of bacteria. There was only one case in which the lesion was dominated by a single bacterial genus (marked as sample CA085, tooth 47 in the figure). This exceptional instance involved *Lactobacillus*, which was found to represent 99% of the RNA-based population in a hidden dentin cavity (55% of the reads had maximal similarity to *Lactobacillus gasseri*, but three other species of lactobacilli were present). However, all of the other samples showed diverse bacterial compositions that varied dramatically between individuals, even within the same carious lesion type (Figure 1). The estimated bacterial diversity was lowest for enamel carious lesions, with an average of 177 species-level phylotypes per sample, and highest for 'open' dentin cavities that were exposed to the oral cavity, which had an average of 251 species per sample. This suggests that open dentin cavities have an input of microorganisms from saliva, even if the biofilm formed on top of the lesion is eliminated from the sample, as was the case here. 'Hidden' dentin cavities, which have almost no contact with the oral cavity except for a minimal lesion through the enamel, had an estimated number of species-level phylotypes of 201 [20]. The observation that multiple species are detected by their RNA in carious lesions unequivocally demonstrates that they are alive and supports the concept that consortia formed by multiple microorganisms act collectively to initiate and expand the cavity. It has been shown that several oral species can act synergistically to increase their pathogenic effect [21]. In a fascinating example of metabolite cross-feeding, *Streptococcus gordonii* was shown to increase the virulence of *Aggregatibacter actinomycetemcomitans* in an animal abscess model [22]. Mixed cultures of *S. mutans* and *Veillonella alcalescens* were also found to produce higher acid levels than biofilms containing only one of these species [23]. In addition, *Veillonella* may favor acid-producing bacteria in caries through nitrate reduction [24], given that low concentrations of nitrite killed several cariogenic organisms. Finally, it must be remembered that not only bacterial interactions but also bacterial–fungal associations can be vital for promoting virulence in disease-associated consortia [25]. For instance, *S. gordonii* modulates biofilm formation in *Candida albicans* and *Candida* load influenced oral bacterial diversity and antibiotic resistance (reviewed in [26]). Also, viruses may play a vital role in shaping microbial populations [27], but this has been poorly studied in oral environments [27].

In the putative list of caries-associated bacteria revealed by this metatranscriptomic approach, *S. mutans* accounts for 0.02% of the active microorganisms in hidden dentin cavities, 0.48% in open dentin cavities, and 0.73% in enamel carious lesions [20]. Thus, although there is substantial evidence that *S. mutans* is associated with caries risk, other species clearly arise as main players in the microbial community, including *Veillonella*, *Rothia*, and *Leptotrichia* in enamel caries and *Streptococcus sanguinis*, *Atopobium*, *Schlegelella*, *Pseudoramibacter*, and *Lactobacillus* in dentin caries. Some of these bacteria are poorly characterized, as exemplified by the genus *Schlegelella*, in which the 16S rRNA sequences identified in this and other studies [28] indicate that this caries-associated oral microbe is a different species from the only two isolated organisms in this genus that are currently described, both in nonhuman niches. The polymicrobial nature of carious lesions implies that animal models are probably not representative of human oral disease, especially in cases where single bacterial species are inoculated in the animal [29,30].

A revealing aspect of RNA-based studies is that the composition of active bacteria in initial, enamel lesions appears to be different from that found in more advanced dentin cavities. This observation holds even in cases where enamel and dentin cavities from the same tooth were sampled and analyzed. An example is shown in Figure 2, where the hidden dentin cavity of a molar tooth had high frequencies of *Neisseria*, *Lactobacillus*, *Megasphaera*, and *Rothia*, whereas a non-cavitated enamel caries lesion had high frequencies of *Haemophilus* and *Gemella*. In addition, the streptococci were dominated by *S. sanguinis* in the dentin cavity, while *Streptococcus mitis* was at significantly higher levels in the enamel caries lesion, which also showed a higher streptococcal diversity [20]. A previous metagenomic study demonstrated that

Box 1. Targeting the causative agents of dental caries

DNA-based studies of microbial diversity in the oral cavity have estimated that the human supragingival dental plaque (the biofilm formed on tooth surfaces) contains between 500 and 700 bacterial species [10,12,16]. These estimates are even higher in saliva, probably because this oral fluid is in contact with all niches in the mouth, reaching values between 1000 and 2000 species in stimulated saliva [52]. In carious lesions, however, the number decreases dramatically to 100–200 species-level phylotypes, both in initial, enamel caries lesions and in dentin or deep-dentin cavities [14,15,35,53], but because these studies are based on PCR amplification of DNA many of the organisms detected may be inactive and not contributing to lesion progression. The recent RNA-based data (see Figure 1 in main text) identify bacteria that are actively involved in translation processes, narrowing the list of caries-related organisms to 40–160 per sample, which are presumably those active in individual cavities. It has been assumed for years that the bacteria involved in the disease should also be present in saliva, which has been the preferred oral sample collected in etiological and epidemiological studies of dental caries due to its noninvasive nature (see, for instance, [54,55]). However, when saliva, dental plaque, and carious lesions from the same individuals are analyzed, it is readily observed that saliva is not representative of the bacterial diversity located at the disease site (Figure I). The microbial composition of the enamel lesion under study (orange circle) appears to be dominated by *Veillonella*, *Fusobacterium*, and *Porphyromonas*, whereas in the saliva sample (blue, outer circle) from the same individual *Streptococcus*, *Neisseria*, and *Prevotella* are the genera found at the highest proportions. This is in agreement with other studies that strongly suggest that saliva samples are not appropriate for studying the microbiology of oral diseases [52,56]. Although the dental plaque samples (green circle) are more similar in bacterial composition to that found in their respective carious lesions, several genera decrease and other increase in proportion in the cavity, as a consequence of the more specialized niche. Thus, even dental plaque will not accurately show the bacterial communities responsible for dental caries and the data strongly recommend the use of carious lesion samples with RNA-based approaches (pink, inner circle) to determine the active etiological agents of the disease.

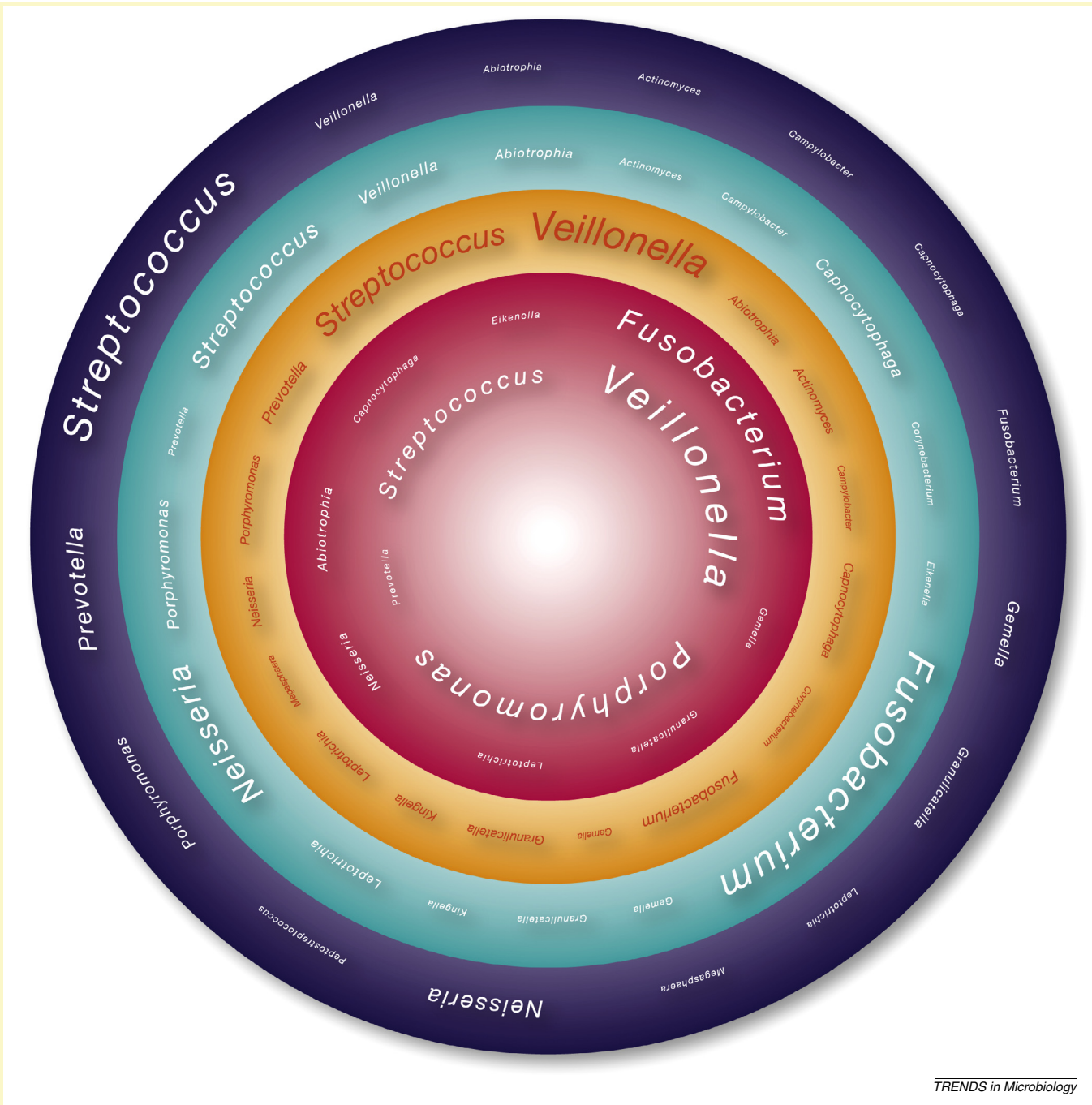

Figure I. Bacterial composition of different oral samples from the same individual. Each ring corresponds to DNA extracted from drooling saliva, dental plaque from sound surfaces, and an enamel carious lesion, as well as from RNA from the same lesion (inner circle). Font size is related to the proportion of each taxonomic group in the sample.

enamel caries bacteria have an over-representation of dietary sugar-fermenting genes, whereas dentin caries organisms are enriched in genes involved in the metabolism of human-associated glycans [14]. In addition, enamel caries microorganisms are extremely rich in adhesion molecules whereas the microbial community in dentin caries contains a remarkable arsenal of proteases to degrade dentinary tissue, including collagenases, dipeptidyl peptidases, serine proteases, glycoproteases, matrix metallopeptidases, and aminopeptidases. The environments that the two cariogenic microbial consortia inhabit are also reflected in the most common stress genes: oxidative stress genes in both tissues, acidic stress genes in enamel lesions, and osmotic stress genes in dentin cavities [14]. Thus, the data clearly demonstrate that caries microbiology is tissue dependent and does not a have a unique etiology, which has important consequences for disease prevention.

Preventing tooth decay

The fact that caries-associated bacterial consortia vary at different stages of disease progression and are dissimilar in different individuals suggests that the same functions may be performed by different sets of microorganisms. This appears to be a general trend in metagenomic studies of the human microbiome, where radically different taxonomic compositions give rise to surprisingly similar functional

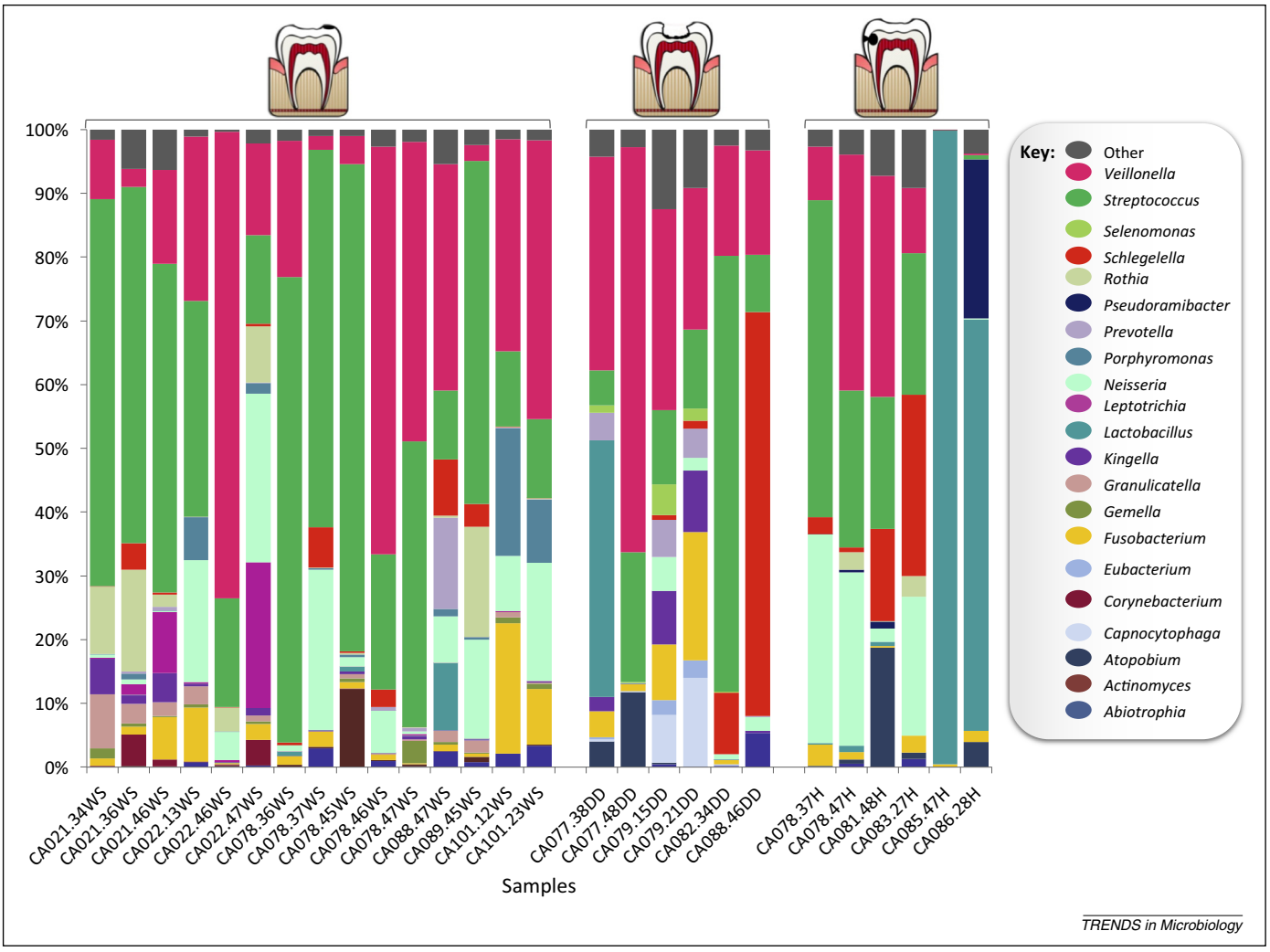

Figure 1. Metatranscriptomic profile of caries lesions. RNA was extracted from initial, white-spot enamel caries (WS), the deep layer from open dentin cavities (DD), and hidden dentin cavities (H). After cDNA construction, bacterial taxonomy was obtained by Titanium-plus pyrosequencing of 800 bp PCR products encompassing hypervariable regions V1 through V4 and using the Ribosomal Database Project (RDP) classifier. Over 4000 reads per sample were obtained, giving information about the active microbial community. The two numbers to the right of the patient code indicate the tooth from where the sample was obtained. Drawn from data in [20].

profiles [31]. Thus, even if determining the etiology of a microbe-mediated disease could be considered the first step in designing preventive strategies, knowing the specific microbial composition will not suffice to understand and combat the disease. Initial hypotheses about the underlying microbiology of oral diseases, which focused on determining the specific causative agents, gave way to ecology-based propositions where the disease is seen as the output of a skewed microbial community due to environmental changes [32]. If the microbial composition of carious lesions is so variable and the combinations of possible consortia so numerous (Figure 1), is it relevant to know a list of cariogenic players? The ecological hypothesis of dental caries considers that focusing on the metabolic output of microbial communities can be the clue to understanding and controlling the disease, regardless of the microorganisms involved in the process [33], and different omic approaches have been initiated to understand the holistic functional output of dental plaque [17,34]. From an applied viewpoint, we believe that the detection of disease-associated metabolic profiles would be more informative than microbial composition in predicting risk of oral diseases and could have potential diagnostic value.

Thus, the change in paradigm in the etiology of tooth decay must be translated to appropriate therapies [35]. Given the polymicrobial nature of dental caries, we predict that diagnostic and preventive strategies directed toward specific bacterial species will not be universally effective. These include diagnostic kits of caries risk assessment, which are traditionally focused on culture counts of mutans streptococci and lactobacilli [4] whereas the data show that the former are found at extremely low proportions even within cavities and the latter are found only in several dentin cavities and not in initial, enamel caries lesions (Figure 1), confirming that this species is not involved in the initiation of caries [36]. Regarding preventive or therapeutic approaches, passive immunization strategies such as the use of synthetic antibodies directed against *S. mutans*-specific antigens [37] are predicted to be inefficient given the multispecies nature of caries initiation. Active immunization strategies (i.e., a caries vaccine) are being explored mainly against mutans streptococci antigens [6,38] and even the multispecies approach that

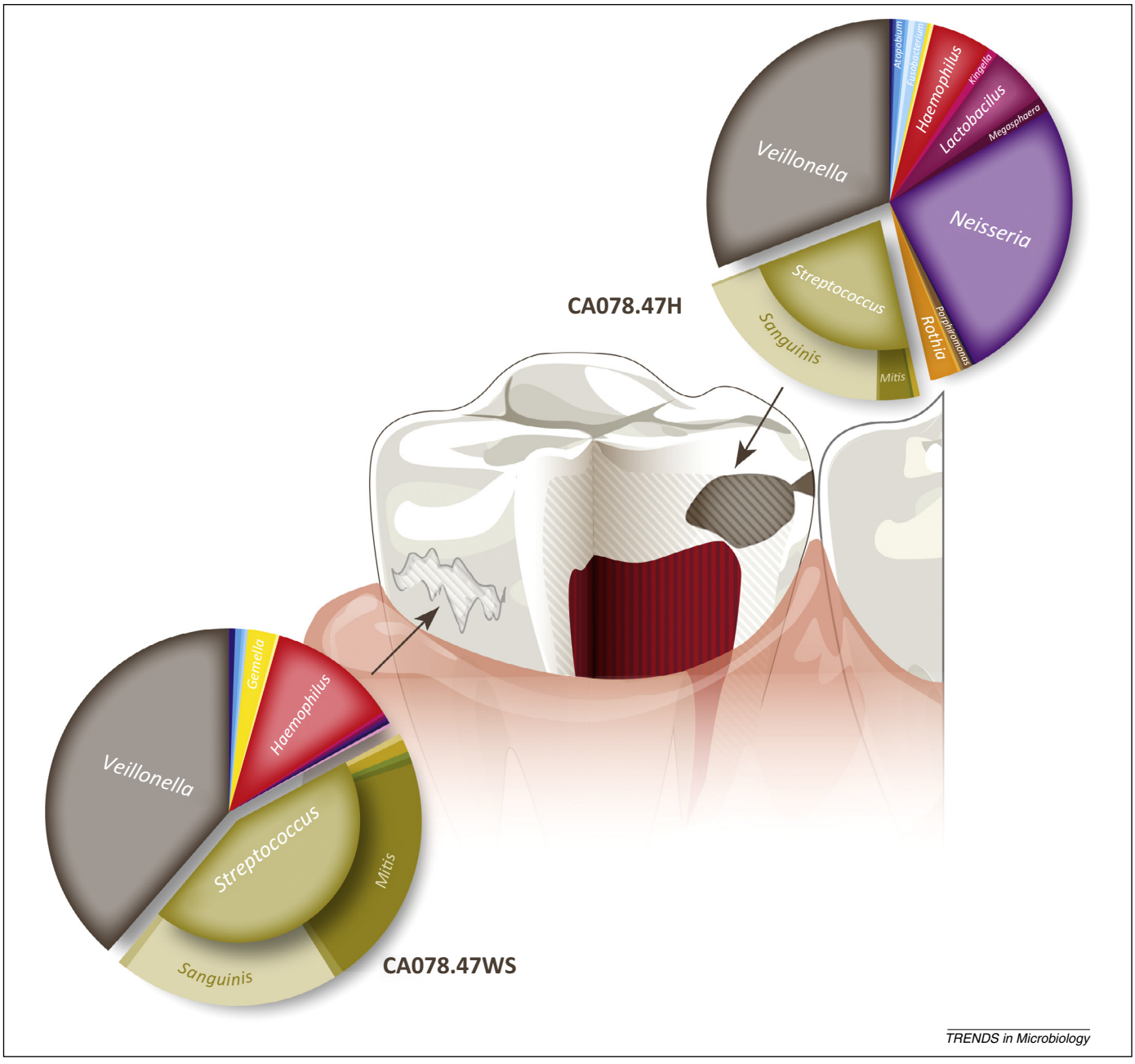

Figure 2. The bacterial composition of caries is tissue dependent. A sample from an initial, white-spot enamel caries (WS) and a hidden dentin lesion were taken from the same tooth (the second molar from the fourth quadrant). The lack of contact between the two lesions was assessed radiographically. The active, RNA-based bacterial taxonomy was obtained by pyrosequencing of long PCR products encompassing hypervariable regions V1 through V4. Drawn from data in [20].

was later proposed [39] may not suffice if a key, universal bacterial repertoire is not present in caries development. Thus, future research should focus on intercepting functions related to disease initiation and progression regardless of which is the bacterial player behind the role (Box 2). Tentative examples of functions to be inhibited in future caries preventive strategies might include quorum sensing and other intercellular communication signals, carbohydrate-fermenting pathways, intermicrobial and microbial–human adhesion compounds, bacterial–immune system crosstalk molecules, and pH-regulating particles. Disruption of adhesion molecules may be a fruitful strategy to either prevent or modulate microbial attachment to the tooth pellicle or between key players in biofilm formation

Box 2. Outstanding questions

- Is the functional output of a microbial community more important than its taxonomic composition in terms of understanding a polymicrobial disease?
- Are immunization and antimicrobial strategies effective in preventing oral diseases?
- Are we treating polymicrobial oral disorders as infectious single-species diseases?
- What non-antimicrobial therapeutic strategies are feasible to prevent polymicrobial diseases such as dental caries?
- Given that the human microbiome contains not only bacteria but also fungal and viral organisms, how common are polymicrobial (as opposed to polybacterial) diseases?

Table 1. Key features of single-species and polymicrobial oral diseases

Etiology	Type of disease	Present in health	Virulent behavior	Immune response	Treatment
Single species	Infectious	No (invasive)	Pathogenic	Active	Antimicrobial
Polymicrobial	Dysbiosis	Yes (commensal)	Pathobiont	Basal	Restore balance

[40]. This is based on the premise that interference in the development of the oral biofilm caused by impairing the tooth attachment of early colonizers or their ability to recruit other key players during biofilm formation would affect the entire process and avoid the presence of cariogenic or periodontal pathogens. For instance, *Fusobacterium nucleatum* is a promising candidate given its capability to coaggregate with both early and late colonizers of the oral biofilm [41]. Controlled (e.g., at subinhibitory levels), limited, or specific use of antibiotic therapy has also been proposed [42].

Another alternative to modulate plaque development without making use of an antimicrobial strategy is the use of probiotics. The introduction of strains specific to the biofilm could theoretically modify key microbial interactions or buffer cariogenic effects, favoring a healthy, balanced ecosystem. However, current clinical trials with probiotic treatment have not been particularly effective in reducing caries rates [43]. A reason for this can be related to the use of single species in probiotic approaches. Not only pathogens act in groups: balance-promoting commensals probably need multispecies clusters to their facilitate settling on the biofilm and to provide beneficial effects. Perhaps for this reason, in the human gut, fecal transplantations have been shown to be effective in treating *Clostridium difficile* infections, colitis, and irritable bowel syndrome, suggesting that an already-formed community settles more effectively in the recipient niche and has a higher probability of conferring a positive effect [44]. However, the practical application of oral transplants would have important drawbacks in daily use, ranging from lack of reproducibility to social rejection of patients or lack of control in the dose and composition of the administered material. A potential solution could be the identification of microbial species that may form stable, cooperative consortia in healthy individuals and that could be used to design probiotic complexes with preventive purposes. In addition to these controlled oral transplants, prebiotics – substances that promote the growth of specific microbial consortia – are a promising option but have unfortunately been only modestly explored [45]. Future metatranscriptomic, proteomic, or metabolomic approaches [46] may provide clues to identify key molecules that could interfere with dental caries initiation and progression [17] and could circumvent the complex and elusive polymicrobial nature of this and other oral diseases.

Polymicrobial versus infectious disease

Apart from dental caries, other oral diseases have also been clearly identified as polymicrobial, including gingivitis, halitosis, and root canal infections [47]. A well-studied case is periodontitis, where even the initial three-species cluster proposed to underlie disease development (the so-called 'red complex' of periodontal disease) has proved to be insufficient to explain its etiology [48]. An expanding list of polymicrobial diseases is emerging outside the oral cavity, ranging from abscesses to bacterial vaginosis, diarrhea, rhinosinusitis, and chronic infection in the lung of patients with cystic fibrosis [49]. The common detection of pathogens present at low levels in health in most polymicrobial oral diseases strongly suggests that they cannot be considered to have an infectious nature and that the microbial causative agents are better described as pathobionts [50,51]. These organisms are therefore resident bacteria with the potential to cause disease, and under balanced conditions the immune system does not have an active response against them (Table 1). From an applied viewpoint, we believe that antimicrobial treatments will be unfruitful in healing or preventing oral polymicrobial diseases and strategies directed toward modulating microbial interactions and/or their functional output should be further developed.

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