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# Salivary Biomarkers for Caries Risk Assessment

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## Abstract

Saliva contains various microbes and host biological components that could be used for caries risk assessment. This review focuses on the research topics that connect dental caries with saliva, including both the microbial and host components within saliva.

Dental caries is recognized as a multi-factorial infectious disease caused by complex interactions among acid-producing bacteria, fermentable carbohydrates and many host factors including saliva.[1] It remains a major health issue in the United States and worldwide with a prevalence of more than 40 percent in young children and about 90 percent in the adult population.[2] Its prevalence rate in childhood is five times higher than the next most prevalent disease, asthma.[3] Despite the dramatic reduction in caries rates over the last decades, it still affects 60 to 90 percent of school-aged children and adults.[4,5] In many countries, severe caries still exists in all age groups,[6,7] which creates huge social and economic burdens.[8]

## Importance of Caries Risk Assessment

Currently, dental caries is mainly treated by restorative approaches, which do not always generate optimal satisfactory results. Caries risk assessment allows for the estimation of the probability of caries incidence, i.e., number of new cavities or incipient lesions in a certain time period, as well as the probability of the changes in the size or activity of caries lesions.[9] An accurate caries risk assessment can identify patients at high caries risk for preventive therapies and improved treatment effectiveness. Therefore, more attention has been given to this topic lately.[10] In particular, the roles of saliva and its biological components have been extensively studied for their possible relevance to dental caries, which is the focus of this review.

## Anti-caries Effects of Saliva

Whole saliva is a complex mixture of oral fluids which is composed of salivary gland secretions, gingival crevicular fluid, expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses, fungi, desquamated epithelial cells, other cellular components, as well as food debris.[11,12] Saliva plays many important roles in maintaining oral health. van Nieuw Amerongen et al.[13] summarized various protective functions of salivary proteins on teeth integrity, including cleaning teeth, protecting against abrasion and attrition, retarding demineralization as well as

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promoting remineralization, rapidly neutralizing acids, and defending the oral cavity from infection.

Saliva provides some real potential in evaluating dental caries risk. Lack of saliva predisposes the development of atypical or unusual dental decay, i.e., cervical, incisal or in cusps tips, as well as radicular lesions.[14] Edgar and Higham[15] categorized the anti-caries effects of saliva as static or dynamic. Static effects are those which may be assumed to be continuous effects exerted on the microbial composition of plaque through antimicrobial or metabolic factors, protective effects of salivary pellicle formation and the effects of salivary electrolytes (including fluoride) in maintaining a supersaturated environment for the tooth mineral. Dynamic effects, on the other hand, are those which are correlated with the flow rate following salivary stimulation and are mobilized over time as indicated by the Stephan curve.[16] These include the clearance of the acid products of plaque metabolism following sugar challenge, and the buffering capacity for restoring plaque pH towards neutrality.[17] Saliva is also known to contain pH-raising factors such as sialin, arginine and urea.[18] Acid produced by acid-producing bacteria following sugar fermentation causes plaque pH values to fall below a critical value resulting in demineralization of tooth surfaces.[19] However, demineralization can be reversed in its early stages. Supersaturation of saliva with calcium, phosphate and fluoride allows remineralization of teeth at this stage.[20]

## Caries-associated Microorganisms in Saliva

Over the past few decades, extensive research has provided significant information regarding the connection between dental caries and salivary bacteria.[21] A primary etiological factor of caries is acid production from dietary carbohydrates by bacteria in saliva and plaque. Potentially cariogenic bacteria are usually present in relatively small quantities in healthy saliva and plaque. However, with biological and environmental perturbations such as the increased frequency of fermentable carbohydrate consumption, conditions of low pH will favor the proliferation of acid-tolerating (and acidogenic) bacteria. When the cariogenic bacteria dominate the saliva and plaque, more acids are produced at even faster rates, thereby enhancing the prevalence of these cariogenic bacteria.[22]

Dental caries-associated oral streptococci are called the mutans streptococci,[23,24,25] with *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) being the predominantly prevalent caries-associated species in humans. Among the physiological traits of mutans streptococci which are most relevant to cariogenesis are their synthesis of extracellular polysaccharides from sucrose which fosters their firm attachment to teeth and promotes tight cell clustering, rapid fermentation of carbohydrates to acids and tolerance to low pH.[24, 25] It has been demonstrated that mutans streptococci can colonize the mouth of pre-dentate infants and are acquired by both vertical and horizontal transmission from human reservoirs, especially mothers.[26,27] The earlier in infancy that high salivary mutans streptococci counts occur, the more severe the caries in the primary dentition.[28,29] Mutans streptococci also exhibit a much higher prevalence and higher proportions in caries-positive subjects than caries-free individuals.[22,30] Among mutans streptococci, *S. mutans* has often been associated with the initiation and progression of dental caries and is generally considered as the principal agent for human dental caries.[23,31] It is frequently isolated from caries lesions and is able to induce caries formation in animals fed a sucrose-rich diet.[32,33] Its prevalence in human caries cases ranges from 70 to 100 percent.[23] In two large-scale microbiological studies, *S. mutans* has been linked to crown caries in children and adolescents,[34,35] and to root caries in elderly patients.[36] By 16S rDNA phylogenetic profiling of dental caries-associated flora, *S. mutans* was found extensively in caries-active subjects.[34,35,37] Suppression of high levels of *S. mutans* in a mother might delay or prevent

the colonization of the organism in her child.[38] In fact, the delayed colonization of *S. mutans* can result in a reduction in dental caries.[39]

Lactobacilli have also been implicated as important contributory species in dental caries,[34,35,37] but their role in initiation of caries is not well supported. They are highly acidogenic and aciduric,[40] but do not avidly colonize the tooth enamel.[41] Instead, they are often cultured from established carious lesions.[42]

van Houte et al. proposed that non-mutans streptococci, including *S. sanguinis, S. oralis, S. gordonii,* and *S. mitis,* could contribute to dental caries as well.[43,44] Among non-mutans streptococci, some are acidogenic and aciduric[45] but less evidence exists of their virulence in experimental animals than either the mutans streptococci or the lactobacilli. In some cases, the data suggest an inverse relationship between the prevalence of non-mutans streptococci and the mutans streptococci, and this relationship is also correlated with caries development.[46,47]

There is also evidence which links *Actinomyces* spp. to the onset of root surface caries.[43,48,49] *Actinomyces* have been shown to induce root surface caries in animals.[50] They can also metabolize carbohydrates but are not particularly acidogenic nor acid tolerant compared to mutans streptococci and lactobacilli. More recently, Mantzourani et al. correlated the prevalence of the family *Bifidobacteriaceae* with cavitated root caries lesions.[51]

The presence of *Candida* species in the oral cavity is usually found to be positively correlated with poor oral hygiene and high carbohydrate intake.[52] Recently, *Candida* species have also been associated with dental caries.[53,54] Studies of oral *Candida* species suggest their cariogenic potential since they exhibit acidogenic heterofermentative properties, especially in the presence of carbohydrates,[55,56] and coaggregation with other bacteria in biofilms.[57,58] Some studies demonstrated that subjects in a caries-active group showed a high frequency of oral candidal carriage compared to caries-free subjects[59] and reported a positive correlation between *Candida* and one-year caries increments.[60]

Therefore, although the mutans streptoococcis are primarily implicated in dental caries induction, other non-mutans microorganisms could also contribute to this disease.

## Caries Risk Assessment Via Analyzing the Levels of Cariogenic Bacteria in Saliva

More than 700 oral microbial species have now been identified, making oral flora one of the most complex microbial communities in the human body.[61,62] Saliva could act as an oral circulating fluid for bacterial transmission and act as a reservoir for bacterial colonization.[63] Bacteria, including anaerobic species, can survive in saliva and utilize salivary constituents for growth.[64,65] There are about $10^8$ to $10^9$ CFU/mL oral microorganisms living in saliva.[23] These salivary microbial species reflect the oral microbial community composition and could serve as a biomarker of the health and disease status of the oral cavity. Saliva allows dental plaque to flourish and also detaches layers of plaque.[66,67] Therefore, bacteria can also be released from plaque.[68] The level of certain bacterial species in saliva can reflect their presence in plaque.[69,70] Previous studies have shown a significant correlation between the salivary concentration of mutans streptococci and their proportions in plaque.[21,71] The levels of cariogenic species in saliva have been investigated as a potential tool for caries risk assessment.[21,24,46,72]

### The Levels of Salivary Mutans Streptococci and Lactobacilli

Many studies have demonstrated that increased proportions of mutans streptococci and lactobacilli in saliva are correlated with increased caries initiation and progression,[21,23,30,73] as well as the presence of root caries.[74] Thenisch et al. summarized 981 reports assessing the association of mutans streptococci and caries in preschool children and concluded that the presence of mutans streptococci in the saliva of young caries-free children appears to be associated with a considerable increase in subsequent caries risk.[75] Regarding the relationship between early childhood caries (ECC) and mutans streptococci, Parisotto et al. also undertook a systematic review and concluded that the salivary mutans streptococci level is a strong risk indicator for ECC[76]. Subjects with multi-surfaced restorations had significantly higher levels of salivary mutans streptococci and the potential for continued caries activity when compared to those without restorations and are caries-free.[77] Less convincing data are available relative to the possible association between salivary lactobacilli levels and caries onset.[24] Lactobacilli likely do not play any significant role in the initiation of dental decay. However, once a lesion has been established, its proportions were seen to increase.[78] The level of salivary lactobacilli appears to reflect sugar consumption by the host.[79] Therefore, salivary lactobacilli level could be indirectly related to caries progression.[80]

As for the predictive threshold of salivary mutans streptococci and lactobacilli, no absolute values for high or low values have been established. For example, Krasse and Fure proposed that $10^5$ mutans streptococci per milliliter of saliva could be considered a high value in a person with only a few teeth and no restorations. However, $10^6$ might not be an extremely high value in a person with many restorations.[81] As for lactobacilli, counts of $10^4$ CFU/mL in saliva would be considered low; high values would equal or exceed $10^6$ CFU/mL in saliva.[71]

Studies on sensitivity, i.e., the probability that caries-active individuals have high values for S. *mutans* or lactobacilli, varied from 44 to 71 percent and it is lower than their specificity (56 to 100 percent), i.e., the probability that individuals without new caries or a low caries incidence have low values for these species.[82,83] This suggests that the negative predictive value might be more accurate compared to the positive predictive value. Therefore, salivary mutans streptococcal counts have better predictive value for selecting people who will not develop caries (i.e., high specificity) than for identification of individuals who will (i.e., high sensitivity). Considering the multi-factorial nature of caries, the caries predictive power should increase when other relevant factors such as previous caries experience are included.[84]

The predictive value of salivary levels of mutans streptococci has been evaluated in many studies; however, the results are not consistent. Although some studies found a significant association between salivary levels of mutans streptococci and subsequent caries onset,[85] other studies revealed no clear-cut association between them.[86,87] The observed discrepancies could also be due to the different methods used to detect salivary mutans streptococci. Excellent positive predictive values for *S. mutans* were found for young children ages 2 to 4 years[28] and for children ages 12 to 13 years.[82] The prediction of low caries risk by salivary mutans streptococci and lactobacilli counting appears to be more reliable than for estimating high caries risk.[21,88] Therefore, salivary mutans streptococci and lactobacilli counting might not be the sole predictor for caries and multifactorial tests would be more reliable.[72,89,90]

Both Larmas[71] and Messer[91] suggested that salivary mutans streptococcal tests be used for pre-selection of patients for dental examination, demonstration of cariogenic infection, evaluation of the effectiveness of chemotherapeutic rinses and providing for an objective

measure of treatment outcomes. As for salivary lactobacilli tests, it was proposed that these be used for planning recall intervals, for evaluating sucrose consumption and sometimes for those medically compromised patients and patients with open carious lesions or orthodontic bands.[71,91]

### The Levels of Other Salivary Caries-associated Bacteria

The predictive power of salivary levels of non-mutans streptococci or *Actinomyces* for caries initiation and progress has not been evaluated rigorously and such an association remains equivocal.

The role of salivary yeasts in caries risk assessment has not been studied extensively but these organisms may contribute to overall microbial acid production,[56] and the associations between caries increment and salivary *Candida* could be observed in children,[92] suggesting that salivary yeast levels could be a potential caries predictor in children. Pienihäkkinen[93] proposed that salivary *Candida* levels had better caries predictive power than salivary lactobacilli levels. A salivary yeast test could be used for confirming the hypo-salivation status of a patient and for evaluating the effectiveness of anti-fungal therapy.[71]

## Caries Risk Assessment Via Analyzing Host-related Factors in Saliva

### Salivary Flow Rate

The half time for saliva clearance is much shorter than the time required for oral bacterial cell division. Therefore, these bacteria cannot survive in the mouth unless they have the ability to bind to teeth or the oral mucosa.[94] In the mouth, there is an equilibrium between the number of free bacteria in saliva and the number bound to the teeth or to oral epithelial cells. Low salivary flow rate is a risk factor for caries incidence.[95] The most common alterations in salivary flow rate involve reduced secretion, which may be influenced by medications, pathological changes in the salivary glands, and age, etc.[96,97,98] It is considered a potential risk factor when the unstimulated salivary flow rate is lower than 0.30 mL/min[71,99,100] and the stimulated salivary flow is lower than 0.7 ml/min.[101]

### Salivary pH and Buffer Capacity

Previous studies have shown larger quantities and faster rates of acid production in caries-active individuals than that in caries-free individuals.[23] The quantitative assessment of resistance to pH changes is referred to as buffer capacity. There is reasonably strong evidence to indicate that salivary buffering capacity protects the tooth from dental caries.[102] Low buffering capacity is usually associated with caries development because of its impaired neutralization of plaque acids and reduced remineralization of early enamel lesions.[103,104,105] Furthermore, an association between low caries levels and high salivary buffering capacity has been also demonstrated.[106,107] Individuals with a high salivary buffer capacity are often caries-resistant.[71]

### Salivary Proteins

Mandel et al. found no difference in parotid saliva protein levels between caries-free and caries-active adults.[108] However Balekjian et al. observed that a caries-rampant group exhibited a significant reduction in the salivary level of basic proteins and a significant increase in amylase compared to a caries-free group.[109] There are also studies that suggest that some proteins in saliva from caries-active and caries-free individuals may have different levels of biological activity.[110,111,112,113] Salivary proteins from caries-active individuals were consistently found to support better growth of *S. mutans* or *S. sanguis* than comparable secretions from caries-free subjects and had a much greater potency for promoting saliva-mediated adherence and lower capacity to induce saliva-mediated aggregation.

Salivary mucins play a major role in the health of the oral cavity.[11,114] MUC7, one of the predominant mucins in saliva, has been reported to interact with several strains of streptococci by promoting their agglutination.[115,116] Diminished levels of MUC7 were found to be significantly associated with elevated *S. mutans* titers, which raises the possibility that dramatically reduced levels of MUC7 might serve as an important predictor in caries risk assessment for older adults.[117]

There are contradictory results in terms of finding a relationship between caries prevalence and salivary proline-rich proteins (PRPs).[118,119,120,121] Salivary glycoproteins participate in the formation of the acquired enamel pellicle, whose constituents will influence initial microbial colonization on tooth surfaces and may therefore affect the microbial composition of plaque. Specific oligosaccharides of salivary glycoproteins could either facilitate bacterial attachment and colonization at the surface of teeth or protect against colonization by promoting agglutination and removal of free bacteria. Based on the pattern of genetically determined oligosaccharides present on salivary glycoproteins, Denny et al. developed a new saliva test for caries risk assessment.[122] They found that the levels of selected oligosaccharides correlated with caries incidence in young adults.

Low salivary levels of alpha-defensins HNP1-3 may represent a biological factor that contributes to caries susceptibility in children.[123,124] Using a proteomic approach, Rudney et al. suggested that salivary levels of statherin and cystatin S may be potential risk indicators for caries development.[125] Higher levels of statherin and cystatin S were detected in caries-free children.[126]

Salivary IgA antibody responses to mutans streptococci can be observed in early childhood.[127] The levels of specific secretory IgA (SIgA) showed a relationship with caries risk, and the literature is nearly equally divided for and against an anticaries role for specific SIgA.[102] As for salivary innate non-immunoglobulin factors, none of the salivary antimicrobials (lysozyme, lactoferrin, total peroxidase activity, hypothiocyanate and thiocyanate) has sufficiently strong association to caries initiation and progress.[102,128] However Mungia et al.[129] reported an association between caries experience and the concentrations in submandibular or sublingual gland saliva of lactoferrin, albumin, lysozyme, mucins and cystatins. They also indicated that changes in saliva output during ageing correlated with greater caries risk and may be an indicator of caries risk.

## New Tools for Salivary Risk Assessment of Caries

### Salivary Bacteria Counts

**Culture-based methods—**Based on microbiology-related caries-risk predictors in saliva, most of the salivary microbial tests by far have been focused on mutans streptococci and lactobacilli. Culture-based methods are a common way to characterize the proportion of salivary mutans streptococci and lactobacilli on selective media. Gold et al. described a selective medium based on the mitis salivarius bacitracin agar (MSB) for mutans streptococci, which were found to be resistant to bacitracin.[130] However, the major limitation of MSB is its relatively short shelf life with a maximum of one week. This is particularly inconvenient when the plates are used in a clinical setting. Mitis salivarius bacitracin broth (MSBB) was developed by Matsukubo et al. with a longer shelf life. In this medium, the concentrations of bacitracin and sucrose were chosen to obtain distinct characteristic colonies and good colonial adhesion to the glass.[131]

In 1940, Snyder described a simple colorimetric test for the indirect determination of the counts of lactobacilli in saliva.[132] Saliva was added to tubes of a selective (pH 5.0) liquefied agar medium. A change in the color of the indicator brom-cresol-green from green to yellow

after 48 hours of incubation was indicative of more than $10^3$ lactobacilli *per* mL of saliva. A further refinement in the cultivation of lactobacilli was an improved selective medium introduced by Rogosa et al. in 1951.[133] This medium allows for growth of an extended spectrum of oral lactobacilli and is still the basis of modern diagnostic salivary lactobacilli tests.

**Dip-slide Methods—**Compared with conventional agar plate techniques, dip-slide tests have been shown to be reliable methods for determining salivary levels of mutans streptococci and lactobacilli.[134,135] At present, all the commercial dip-slide methods for determining the proportion of mutans streptococci in saliva are based on the fact that bacitracin inhibits the growth of all other oral streptococci except mutans streptococci on MSB medium. Currently available commercial kits for detection of salivary lactobacilli are mostly based on Rogosa's medium.

Dentocult SM and LB whose use results in significant correlation with the conventional-selective-culture-based methods have been shown to provide a good microbiological assessment of mutans streptococci and lactobacilli, respectively, in the saliva.[134,135,136] Dentocult Strip Mutans Test and Caries Screen SM (Orion Diagnostica, Espoo, Finland) are other simple diagnostic tests allowing for gross enumeration of salivary mutans streptococci outside of a bacteriology laboratory under both clinical and field conditions.[134,137] Based on Nickerson medium, a dip-slide system, Oricult-N, is also available for measuring oral yeast infections.[96]

**Molecular Methods—**Assessment of caries risk undoubtedly would benefit from newly emerging technologies. More sensitive DNA-based methods including checkerboard DNA-DNA hybridization, genomic fingerprinting, 16S rRNA gene cloning and sequencing, or T-RFLP are also being utilized in identification and classification of dental caries microbiota.[138,139,140] Polymerase chain reaction (PCR)-based bacterial identification can detect a large array of microorganisms in saliva and provides accurate measurements of the known cariogenic species in saliva.[141] The real-time quantitative polymerase chain reaction (qPCR) technique was found to be more sensitive for enumeration of *S. mutans* in saliva compared to the traditional culture-based methods.[142] Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiling and species identification could serve as a community-based molecular technique and allow for the study of the oral bacterial community structure associated with severe dental caries.[143] In addition to intact microorganisms, DNA and RNA released from microorganisms also exist in saliva. Oral streptococcal 16S rRNA/rDNA was identified in the liquid phase of saliva,[144] which suggests a new possibility for oral pathogen detection since the liquid phase of saliva could be directly used for 16S rRNA/ rDNA detection without requiring bacterial cell isolation.

The availability of high-throughput DNA sequencing technology together with the rapid expansion of bacterial genome data has now made it feasible to identify the primary bacterial residents in saliva.[145,146,147] Currently, human oral microbiome studies are still in their infancy and large-scale projects are in progress. Their results should become available in the next few years. It is anticipated that such high-throughput sequencing will assist in identifying potential cariogenic species that may not have been detected using currently available technologies such as 16S rRNA analysis. Recently, one group in China investigated salivary microbiota in both caries-active and normal human populations by cross-validating 16S rRNA gene amplicon-based and whole-genome-based deep-sequencing technologies including 454 pyrosequencing and Solexa sequencing.[148] Its findings raised the possibility of exploiting salivary microbiomes as diagnostic markers of caries.

Another enabling technology for salivary cariogenic bacteria detection is the monoclonal antibody (MAb) technique. Different bacteria present unique surface proteins and polysaccharide structures on the cell surface. MAbs can be raised against these structures and detect the corresponding bacterial species with very high specificity and sensitivity. These antibodies can be linked to various detection systems, such as fluorescent, colorimetric or coagglutination reagents. MAb-based detection methods allow a rapid and accurate way to quantitatively measure cariogenic bacteria. They have significant advantages compared with traditional culture growth assays or PCR techniques. By linking fluorescent dyes to these MAbs, researchers can track bacterial species *in situ* and in real-time. MAbs against the cariogenic species *S. mutans, Lactobacillus casei* and *Actinomyces naeslundii* with 91 percent sensitivity and 96 percent specificity have been developed in our laboratory.[149,150,151] These MAbs were conjugated to different fluorescent dyes and can quantitatively and accurately detect cariogenic bacteria in saliva.[152] Matsumoto et al. also developed an antimutans streptococci MAb.[153]

### Assaying Host Factors

Salivary flow rate can be measured in the resting or stimulated states. The usual salivary collection methods include a draining method using a Proflow Sialometer, a spitting method, a suction method, swab or absorbent methods and the use of a salivette.[154,155] The Schirmer tear test is also used in salivary measurements.[156] Salivary flow rates can be stimulated by a range of oral and physiological stimuli. Chewing paraffin wax is the most common saliva stimulating method.

At present, commercial dip-slide kits that provide for measurement of salivary flow rate, salivary pH and buffering capacity are available for convenient and rapid clinical tests. Saliva-Check (GC America, Alsip, Ill.) is a salivary testing kit that tests for hydration, salivary consistency, resting saliva pH, stimulated saliva flow and pH, and saliva buffering capacity.[157] The Fosdick calcium dissolution test can measure the quantities of powdered enamel dissolved in four hours by acid formed when the subject's saliva is mixed with glucose and powdered enamel.[158,159] Both Wach's[160] and Rickles' tests[161] could determine acid production in a saliva-sugar mixture. Some researchers utilized optical spectroscopic sensors to monitor the bacterial-mediated acidogenic-profile of saliva and found that the sensors were able to detect significant differences in the salivary acidogenic-profiles between subjects of different caries status, which highlighted the possibility that optical spectroscopic sensors might be used as a point-of-care testing tool for caries-risk assessment in children.[162] Salivary buffer capacity can be measured by the Dentobuff method,[163] in which a dip-slide is coated with chemical indicators and immersed in the saliva. The resulting color is indicative of the capacity of the saliva to buffer acids and bases. More recently, a Dentobuff strip (Orion Diagnostica, Espoo, Finland) has also been devised for the same purpose.[164]

## Perspectives

Dental caries is a multi-factorial infectious disease that involves complex interactions among acid-producing bacteria, fermentable carbohydrates and many host factors. Interestingly, almost all these components could be detected in saliva, making saliva-based caries risk assessment a real possibility.

We expect that ongoing innovative research and development will have a significant impact on dental caries prediction and control. We envision that in the future, treating dental caries will be an evidence-based dental practice emphasizing the triple-pronged approach of early detection, effective and sustainable treatment and prevention. Specifically, detection of microbial and host-related caries risk factors can become routine. This approach will help

clinicians to reinforce the concept of dental caries as an infectious process and will facilitate immediate, evidence-based treatment decisions.

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