

# CHAIRSIDE TESTING FOR CARIOGENIC BACTERIA: CURRENT CONCEPTS AND CLINICAL STRATEGIES

LAURENCE J WALSH<sup>1</sup> AND ANNETTA KL TSANG<sup>1</sup>

## Abstract

Modern approaches to dental caries such as STEM (System for Total Environmental Management) are based on a whole-of-patient and whole-of-biofilm approach to the disease. There are now multiple lines of evidence which indicate that dental caries is a multi-pathogen disease and that mutans streptococci may participate but are not critically essential for disease to occur. A number of new strategies for controlling caries risk are currently being explored, based on the modern biofilm concept. Clinical diagnostic kits which examine plaque or saliva for features of the biofilm which are surrogates (markers) of the disease process can be useful in clinical patient management since these can be undertaken at chairside.

## Introduction

Dental caries can be defined as a diet- and saliva-modified bacterial disease.<sup>1</sup> The key microbial feature of dental caries is a dietary carbohydrate-induced enrichment of the normal oral flora with bacteria that are both acidogenic (acid producing) and aciduric (acid tolerant) located within a dense biofilm, rich in extracellular polysaccharides, which maintains a favourable environment for microbial community and protects it from physical and chemical assaults.

The biofilm which causes dental caries arises from the normally thin dental plaque biofilm which is present continuously on hard surfaces in the oral cavity, when this is subject to particular ecological pressures related to intake of fermentable carbohydrates and subsequent production of organic acids by bacterial fermentation. Within the dental plaque biofilm, bacteria inhabit a diverse range of ecological niches ("habitats"), and exist not as isolated species but in complex physical and metabolic synergistic relationships with other species, which provides metabolic advantages to each.

Within a cariogenic biofilm, factors affecting bacterial growth and metabolism include:

- Water – which is a source of hydrogen and oxygen. This is delivered throughout the biofilm by water channels which develop as part of the biofilm structure.
- Carbon - from carbon dioxide and from carbohydrates.
- Organic nutrients - Carbohydrates, proteins (peptides), and amino acids. Bacteria require amino acids for the protein synthesis which is essential for their replication by

binary fission. The breakdown and metabolism of certain amino acids such as arginine can alter the local pH, as will be discussed further below. Various peptides (termed bacteriocins) produced by bacteria can suppress the growth of other non-compatible species, e.g. bacteriocins from *Streptococcus salivarius* and *Streptococcus sanguis* can suppress *Streptococcus mutans* and the periodontopathic organism *Actinobacillus actinomycetemcomitans*, respectively.

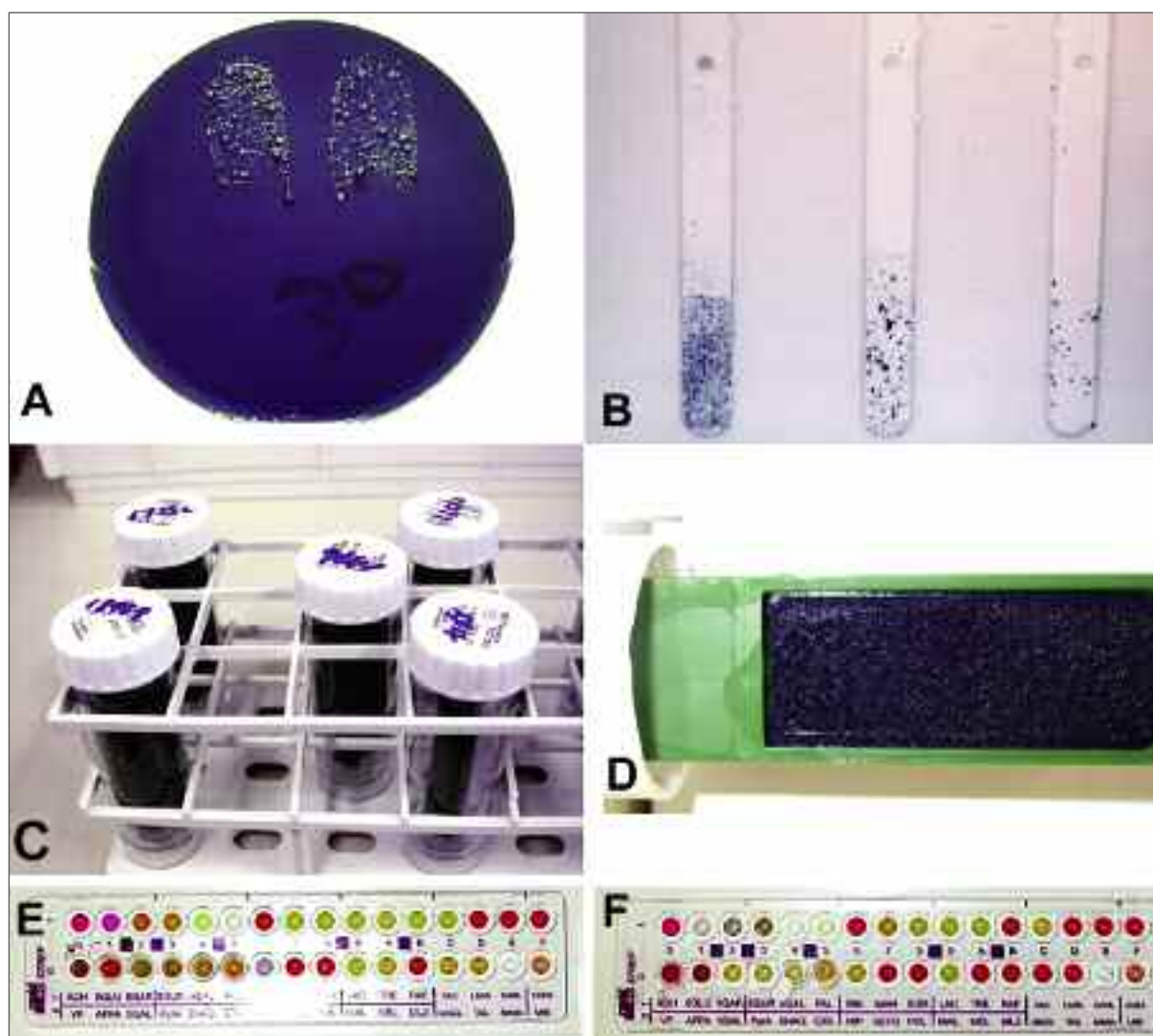
- Inorganic nutrients - e.g. magnesium, nitrogen, sulphur, potassium, phosphate, as well as selenium and other trace elements
- Environmental factors – Temperature, pH, and Redox potential (eH). The falling levels of pH (with acid production) and eH (with thickening of the plaque) help provide ecological niches deep within the plaque for highly fermentative facultative species such as the mutans streptococci (MS). Acids produced deep within the plaque not only demineralize enamel, but suppress the growth of bacterial species which are not aciduric. The shift toward aciduric organisms also promotes an increase in the proportion of lactobacilli, which are highly acidogenic in their own right, producing lactic acid from glucose fermentation. Although lactobacilli are not regarded as important in the initiation of caries, their presence in large numbers indicates that the necessary environmental conditions for producing dental caries exist.<sup>2-3</sup>
- Salivary factors - resting and stimulated flow, pH, fluoride, calcium and bicarbonate levels; salivary anti-bacterial properties (antibodies, lysozyme, lactoferrin, peroxidase).<sup>1,4</sup>
- Tooth factors - anatomical features such as hypoplasia and other surface defects (which are influenced by maternal and peri-natal health and nutrition), and fixed orthodontic

1. The University of Queensland School of Dentistry

Address for correspondence:

Prof LJ Walsh

School of Dentistry, The University of Queensland, Turbot Street  
Brisbane QLD 4000 Australia



**Figure 1:** Various cultural tests used for mutans streptococci. **A,** Tongue blade imprint onto MSB agar. This was a common method used in the 1980's. **B,** "Strip mutans" test strips showing (from left to right) high, medium, and low levels of MS. This was a popular test in the 1990's. **C,** Contemporary (2008) version of the Dentocult tests with separate media for MS and lactobacilli, known as the CRT. **D,** Dentocult SM test strip from the CRT after 72 hours, showing multiple colonies in a saliva sample from a high caries risk patient. **E,** Biochemical test panel (Rapid ID32 Strep) results for *Streptococcus mutans*. **F,** Identical panel with *Streptococcus sobrinus*. Differences between biochemical results allow clear differentiation between these two species.

appliances, which provide increased surface area for retention of the biofilm.<sup>5</sup>

Manipulating the physical, ionic, and metabolic factors which modulate the properties of the biofilm provides a powerful approach to caries prevention and control.<sup>6,7</sup> However, the question must be asked, are such strategies intended to target just one or two particular species of bacteria, or the entire biofilm?

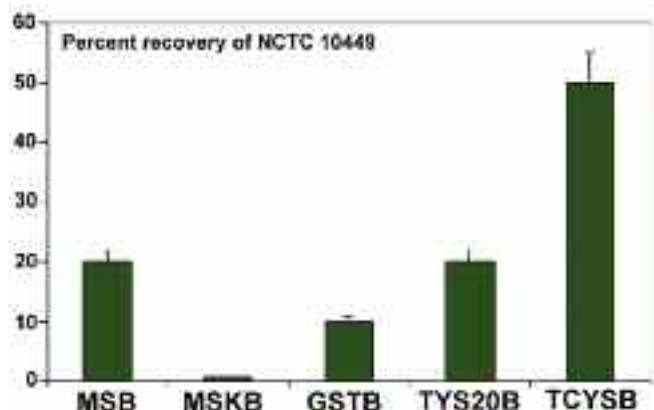
### Is dental caries a specific infection caused by mutans streptococci?

Since the mid-1970's when the "specific plaque hypothesis" was first proposed, the key pathogens in dental caries have

traditionally been considered to be the mutans streptococci (MS) family, with *S. mutans* and *S. sobrinus* thought to be the major initiators of the disease.

Problems with the specific plaque hypothesis for dental caries are many, and in summary form include the following:

- MS levels correlate with caries incidence at the population level, but not necessarily at the individual level. MS counts in saliva and plaque are not linearly associated with caries incidence in an individual patient, despite evidence for a linear caries progression over time.<sup>8</sup>
- MS negative individuals with coronal or root surface caries can be found, albeit at low rates (typically 2 percent).<sup>9,10</sup>
- When used as a survey tool for large population cohorts, culture-based tests for MS tend to give greater negative than



**Figure 2: Bacterial recovery after inoculation of five growth media with 100 million CFU of *Streptococcus mutans* type strain NCTC 10449, expressed as a percentage. Recovery on TCYSB more than twice that of other media, including MSB, the basis of many commercial tests. Data derived from Ref. 82.**

positive predictive values, i.e., they are more effective in identifying healthy individuals (low bacterial counts) than patients with disease who require treatment.<sup>11</sup>

- MS counts alone may vary according to the site sampled as well as the caries risk status. A study in Dundee which compared the frequency of isolation of *S. mutans* and *S. sobrinus* from the saliva of 12 month old infants with and without dental caries collected saliva samples using the tongue-loop method, for subsequent microbiological culture.<sup>12</sup> Of the cohort of 1393 infants, some 39 were diagnosed with caries. *S. mutans* was found at low frequency even in infants with caries, but was isolated more often from those infants with caries compared to those who were caries-free (29.7 vs 9.8%), however differences in the isolation frequencies of *S. sobrinus* (2.7 vs 1.3%) were not significant.
- MS are not unique in the oral flora in being able to secrete glucosyl transferase enzymes or in producing extracellular polysaccharides.
- Many organisms (both bacteria and fungi) in the normal oral microflora are both acidogenic and aciduric, and produce organic acids in vitro following exposure to fermentable substrates.
- MS evokes a host immune response involving both IgA and IgG antibodies, but this is not always protective.<sup>13</sup> Immunization against MS in some animal models reduces the incidence of new carious lesions but does not necessarily eliminate the disease.
- While cariogenic plaque may contain more than 200 million bacteria per mg wet weight, MS counts as a proportion of total plaque bacteria are typically very low (1%).<sup>10,14</sup>
- Patients who have high levels of both major species of MS (i.e. *S. mutans* and *S. sobrinus*) tend to have a higher caries rate than those with only one species, but it is common to

find adult and elderly patients with *S. mutans* only and not *S. sobrinus*.<sup>15</sup>

- There are 300-plus species of bacteria in the non-cultivable normal oral flora in humans (i.e. those which at present cannot be grown in the laboratory, but can be identified by molecular genetic methods), and many of these are found in significant numbers in cariogenic biofilms.<sup>16</sup>
- There is no obligate requirement for MS in terms of generating a pH drop in response to sucrose and other fermentable substrates. Moreover, many bacterial species other than MS can grow and survive at pH values low enough to demineralize enamel, e.g. *Veillonella dispar* and *Enterococcus faecalis*, as well as *Lactobacillus* spp.<sup>17</sup>
- There is substantial variation in the microflora at different sub-sites within approximal dental plaque. As plaque develops in the interproximal region, MS preferentially colonize a specific sub-site below the contact point, at which *S. mutans* is more common than *S. sobrinus*.<sup>18</sup> *S. sobrinus* is rarely found in isolation, and is more often found in association with *S. mutans* (if present at all). However, *S. sobrinus* is the more potent of the two species in terms of acid production and synthesis of both intra- and extra-cellular polymers.<sup>14</sup>
- Non-MS aciduric bacteria such as *Actinomyces naeslundii* are involved in the pathogenesis of root surface caries.<sup>19,20</sup> In root surface caries, neither MS nor *Actinomyces* species secrete collagenases, however proteolytic enzymes originate from other biofilm species such as *Porphyromonas gingivalis*.<sup>21</sup>
- Strains of MS differ in their virulence traits such as the potential to synthesize water-insoluble glucan polymers from sucrose. This is one reason why colonization with MS does not always lead to dental caries activity. Strains which synthesize small amounts of insoluble glucans show reduced adherence within the biofilm and slower accumulation of plaque, with less expression of disease.<sup>22</sup>

A further and major philosophical problem with the specific plaque hypothesis is that MS are normal (commensal) members of the oral microflora, rather than external pathogens. While transmission of MS from mother to child via a spoon was demonstrated conclusively in the landmark studies of Kohler and Bratthall in the late 1970's,<sup>23</sup> the concept of a "window of infectivity" at 26 months of age<sup>24,25</sup> has now been disproven in a series of studies<sup>26-28</sup> which demonstrated that inoculation with mutans streptococci and other oral bacteria occurs within the first three months of life, with repeated exposure from carers (particularly the mother) and siblings through saliva microdroplets. Moreover, the traditional concept that MS lack a



**Figure 3:** GC Saliva-Check SM chairside diagnostic kit based on a solid phase immunoassay, with three monoclonal antibodies specific for *Streptococcus mutans*. A, Components of the kit, showing the test device and the two reagents. B, Addition of the first pH adjusting reagent. C, Addition of the second pH adjusting reagent. D, Agitation of the sample. E, Loading onto the test strip. The liquid moves down the strip by capillary action. F, A positive result with colour reactions seen in both the test (T) and control (C) wells.

suitable ecological niche in the pre-dentate oral cavity, and cannot establish until the primary teeth erupt, has also been formally disproven by studies which demonstrate pre-dentate acquisition, i.e. before hard tissue surfaces are present.<sup>28,29</sup>

Once acquired, the oral flora with respect to dental caries appears to be relatively stable, adding further weight to the premise that so-called cariogens are normal parts of the oral flora. Genotypes (strains) of mutans streptococci are relatively stable within the one individual, and tend to persist for several years.<sup>30</sup>

### Biofilm ecology and its implications

The “ecological concept” of dental caries is based on the view that a catastrophic change in the normal plaque biofilm is responsible for disease.<sup>31,32</sup> The main advantages of this approach<sup>17,33</sup> are that it:

- Deals with the problems in the specific plaque hypothesis, as summarized above.
- Recognizes the possibility that bacteria yet to be cultured can participate in the caries process.
- Aligns with a comprehensive approach to caries as a disease which results from the interplay of host, microbial, lifestyle and behavioural factors.

- Supports a concept of “caries risk management throughout life”, rather than focusing only on “windows of infection” or assumed high risk period. In so doing, it recognizes the clinical reality that caries can develop at any time after tooth eruption.
- Leads to novel and improved strategies for risk assessment, risk reduction and clinical management.

Applying this “ecological catastrophe” concept to the issue of pH within the biofilm, it is clear that the low pH environment generated from carbohydrate metabolism is the major factor responsible for the shifts observed in the oral microflora with high carbohydrate diets. Laboratory studies using chemostat systems in which pH conditions can be manipulated<sup>34</sup> provide useful insights into how the microbially generated pH changes can cause such shifts. In this study, three chemostats were used. In the first, following glucose metabolism, the pH fall was restricted to a minimum value of pH 5.5, while the pH fall was arrested in the other two chemostats at either pH 5.0 or 4.5. When the pH was allowed to fall, the numbers and proportions of *Streptococcus mutans* and *Lactobacillus rhamnosus* increased. Importantly, this increase was directly related to the

**Table 1: Techniques for assessing biofilm behaviour**

|  |
|--|
| <p><b>Biomass (plaque thickness and maturity)</b></p> <ul style="list-style-type: none"> <li>• Disclosing with erythrosin dye</li> <li>• 2-Tone disclosing (GC Plaque-Check)</li> <li>• Fluorescence (KaVo DiagnoDENT; Durr VistaProof)</li> </ul> |
| <p><b>Acid production</b></p> <ul style="list-style-type: none"> <li>• Fermentation test (GC Plaque-Check+pH)</li> </ul>   |
| <p><b>Level of MS</b></p> <ul style="list-style-type: none"> <li>• Cultural assays (Ivoclar Vivadent Dentocult-SM)</li> <li>• Immunoassays (GC Saliva-Check SM)</li> </ul>   |

magnitude of the pH fall. The increase in proportions of acidogenic bacteria was accompanied by a fall in the proportions of acid-sensitive species (such as *Fusobacterium nucleatum*, *Streptococcus gordonii* and *Streptococcus oralis*). Thus, a fall in pH to between 5.5 and 4.5 will allow the enrichment of potentially cariogenic species whilst permitting species associated with health to remain relatively unaffected. However, a further reduction in pH (<pH 4.5) will not only enhance the competitiveness of cariogenic organisms, but will inhibit the growth and metabolism of non-caries-associated species.<sup>17</sup>

In patients with low salivary flow rates (regardless of the cause of this), the concurrent reduction in salivary flow and pH can be expected to provide a more favourable growth environment in which aciduric organisms can flourish.<sup>1,4</sup> While this effect is well recognized for salivary dysfunction induced by medications, irradiation of salivary glands, and salivary gland diseases such as Sjogren's syndrome,<sup>1,35,36</sup> it should also be remembered that the same acidic conditions can be created both by a diet with high intakes of acidic drinks and foods (exogenous acids), and by eating disorders or gastrointestinal conditions (such as chronic gastric reflux) in which the source of the acid is endogenous (hydrochloric acid from the stomach). The latter patients will typically present with dental erosion and occasionally with frank caries.<sup>6,37</sup> The more dramatic and unusual nature of dental erosion may well distract the clinician from realizing the likelihood of greatly increased counts of aciduric bacteria because of their preferential growth under acidic conditions. Several studies<sup>38,39</sup> have reported that patients with eating disorders are more susceptible to both dental caries and erosion, and that such patients with low resting salivary flow rates have very high counts of MS and lactobacilli. Clearly, the preventive program for such patients must be targeted to address the global risk factor of low pH which underpins both dental caries and dental erosion.

**MS levels as a surrogate measure for a cariogenic dental plaque biofilm**

By acknowledging that MS are no longer regarded as sole or

necessarily dominant pathogens in dental caries, it follows that assessment of the dental plaque biofilm should be based on parameters such as acid production by fermentation under conditions of substrate challenge,<sup>6,17</sup> and perhaps by bacterial growth and survival under conditions of low pH. These global assessments will, by definition, be inclusive of all bacteria involved in the caries process, and not only MS. Taking a broad approach to the biofilm ecology recognizes the so-called "insurance hypothesis",<sup>40</sup> i.e. that the biodiversity of the biofilm insures it against declines in function over time, and makes it more resistant to external stressors.

Because of the properties of the cariogenic plaque biofilm, the presence of MS is still useful as a surrogate measure for:

- Adverse changes in the biofilm ecology driven by low salivary pH,<sup>9</sup>
- Behavioural factors such as snacking with sucrose, which lower biofilm pH,<sup>6,17</sup>
- Lifestyle factors which may transfer significant loads of pathogens to infants by salivary microdroplets (such as kissing and on-demand feeding with sweetened bovine milk),<sup>26-28</sup> and
- Development of a plaque biofilm with tolerance of low oxygen environments.

MS are facultative microorganisms, which can tolerate oxygen levels in normal mouth air and in the general atmosphere, but prefer oxygen-poor growth conditions. This feature is essential not only to their survival deep within the plaque biofilm but is also critical in terms of favoured growth sites such as fissures and interproximal spaces.

The ability to thrive in an anaerobic environment is also linked to the ability of bacteria involved in dentine caries to survive in the dentine in lesions that do not have obvious entry points on the enamel surface. For these so-called "occult" or hidden lesions, it is likely that the cariogens gain access to the dentine-enamel junction via lamellae or cracks in the enamel. These lamellae are distributed throughout enamel in both deciduous and permanent teeth. By entering these tooth surface defects, the cariogenic bacteria are at a strategic advantage over the host since they are protected from saliva and its components as well as oral hygiene and other interventions.<sup>41</sup>

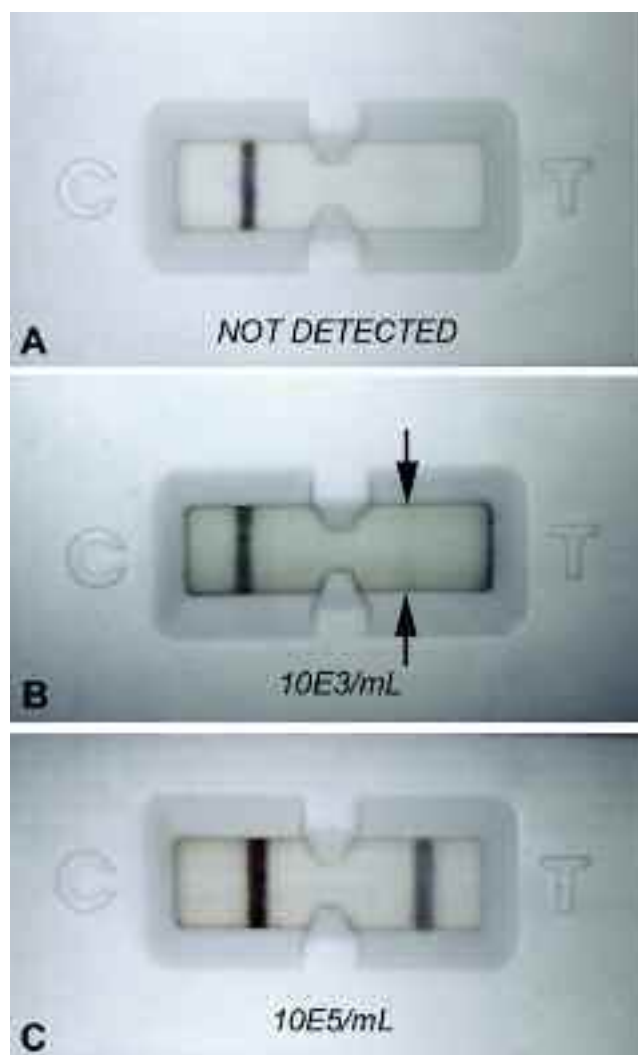
The facultative nature of MS explains in part the interaction between smoking and caries risk. Smoking creates anaerobiosis within the oral cavity, and the low oxygen environment favours the growth of mutans streptococci. There are two additional components involved in the impact of smoking on dental caries. The first is salivary dysfunction, which is the result of the pharmacological effects of nicotine on salivary gland flow.

Reduced flow is linked with reduced pH, which is also a favourable parameter for growth. The second factor is the direct effect of nicotine in the salivary milieu on mutans streptococci. There is now some evidence that nicotine itself can directly affect the growth of *Streptococcus mutans*, with concentrations of nicotine in the order of 0.1-1.0 mmol/L able to stimulate growth, although higher concentrations are inhibitory.<sup>42</sup> A value of 1.0 mmol/L approximates the salivary levels achieved with some tobacco products, including smokeless tobacco.

The tendency for MS to occupy an anaerobic ecological niche has an interesting consequence in terms of periodontal therapy. It is well known that both attachment loss per se and root surface exposure from surgical and non-surgical periodontal treatment are associated positively with root surface caries. The susceptibility for root surfaces for caries after periodontal therapy has been attributed to a loss of the fluoride-rich outer layers of cementum and dentine,<sup>43</sup> however the microbial effects of periodontal therapy should also be considered. Through soft tissue changes such as recession, the available supragingival area for dental plaque biofilm is increased. There is clear evidence that following periodontal therapy, the severity of root surface caries is associated strongly with high salivary counts of MS.<sup>44,45</sup> This effect is compounded in very elderly patients, in whom polypharmacy causes reductions in the resting salivary flow rate and salivary pH, and corresponding increases in both sugar clearance time<sup>15</sup> and levels of MS.<sup>15,46,47</sup>

A longitudinal study which examined the oral flora in patients with severe periodontitis after thorough scaling and root planing in combination with optimal plaque control raises some interesting observations.<sup>48</sup> At baseline and after 4 and 8 months, samples were taken from the saliva, the tongue dorsum and the supragingival interdental spaces. These samples were cultured both aerobically and anaerobically, in order to determine the total number of colony forming units (CFU) per sample as well as the numbers of *Streptococcus mutans* and lactobacilli. Although the total number of aerobic and anaerobic CFU in samples from the tongue and the saliva remained nearly constant over the 8 month period, there were marked increases in the number of *S. mutans* detected in samples from the teeth, despite a decrease in the total number of anaerobic CFU in samples from the teeth, meaning that the relative proportion of the *Streptococcus mutans* had increased dramatically. Thus, although the periodontal conditions improved for all patients, the development of a cariogenic plaque biofilm continued.

This stresses the need for a broad approach to assessing patient risk, and highlights where the overall assessment of plaque biofilm properties can be useful. A range of products



**Figure 4:** Typical immunoassay results obtained in clinical practice and verified by culture on specific media. **A,** Control sample of bacteria-free filtered saliva, showing lack of reaction in the test well, but a positive control result. **B,** Saliva sample from a patient with 1000 CFU/mL is below the caries risk level and does not cause a positive test result. **C,** Saliva sample from a patient with 100,000 CFU/mL gives a clear positive test result.

are now available for this purpose (Table 1). The Snyder test is based upon the fermentation of glucose by bacteria from a sample of stimulated saliva. The resulting acid production over 72 hours lowers the pH of the medium and changes the colour of a pH indicator. This test demonstrates total acid production by cariogenic microorganisms but does not delineate between mutans streptococci and lactobacilli.<sup>61</sup>

Modifications of this same principle, in which the pH-lowering potential of pooled plaque samples was measured in an adapted glucose broth showed some association with caries prevalence,<sup>49</sup> however site-specific tests for fermentation such as the GC Plaque-Check+pH™ test<sup>17</sup> overcome the disadvantages of pooled plaque samples or using saliva samples.

### Measuring MS levels at the chairside

Numerous studies over the past two decades have established a strong link between the presence of pathogenic bacteria at a

**Table 2: Culture-based methods of testing for MS**

| Collection methods                         | Culture media                       |
|--|-------------------------------------|
| Tongue spatula <sup>52,55,57,59</sup>      | MSB <sup>23,52-59,63,64,66,68</sup> |
| Metal spoon <sup>23</sup>                  | MSA or MSA-mannitol <sup>58</sup>   |
| Tongue swab <sup>54,56,60</sup>            | MSA <sup>58</sup>                   |
| Stamp method <sup>56</sup>                 | SB20 <sup>60</sup>                  |
| Plaque swab <sup>60,61</sup>               | MSB Glass <sup>64</sup>             |
| Cariescreen <sup>62,66,67</sup>            | MSB Saccharose <sup>72</sup>        |
| Modified Cariescreen <sup>62</sup>         | MS Sucrose <sup>73</sup>            |
| Glass adherence <sup>63</sup>              | MSS-MUT <sup>74,75</sup>            |
| Dip-slide + MSB <sup>65</sup>              | MUTV <sup>75</sup>                  |
| Toothpick method <sup>68</sup>             | TYC <sup>76</sup>                   |
| Floss method <sup>69</sup>                 | MST <sup>76</sup>                   |
| Microbrush method <sup>69</sup>            | MST Sucrose <sup>76</sup>           |
| Dentocult Strip mutans <sup>56,69-71</sup> | LAPT <sup>77</sup>                  |
|  | MKSB <sup>81</sup>                  |
|  | TSY20B <sup>78,82</sup>             |
|  | TYCSB <sup>78-80,82</sup>           |

This table lists published variations in culture methods and in culture media for MS. Note that there is no relationship between the left and right columns, i.e. different media have often been used with the one sample collection strategy. For details of the methods and culture media, refer to the references cited.

young age and greater caries experience. Both cross-sectional and longitudinal studies of dental caries have established the value of salivary MS as a surrogate marker in caries risk assessment. Thus, while not a sole pathogen, undertaking periodic (e.g. annual) assessments of MS levels may be useful for identifying patients with long-term caries risk in both the primary, mixed and permanent dentitions.<sup>1,50</sup> Today, the range of methods for measuring MS levels includes culture-based tests, metabolic tests, antigenic tests such as direct immunoassays, and molecular genetic methods.

**Culture-based tests**

Culture-based methods for MS and also for lactobacilli have been available commercially for many years, with a wide number of sample collection strategies and culture media being suggested as optimal (Table 2).<sup>51</sup> For culture of oral streptococci, the most common base medium is Mitis-Salivarius agar, or Mitis Salivarius agar with bacitracin (MSB). This is inoculated with stimulated saliva or dental plaque. In most contemporary culture-based tests, this same MSB medium is inoculated with stimulated saliva, which is elicited by chewing

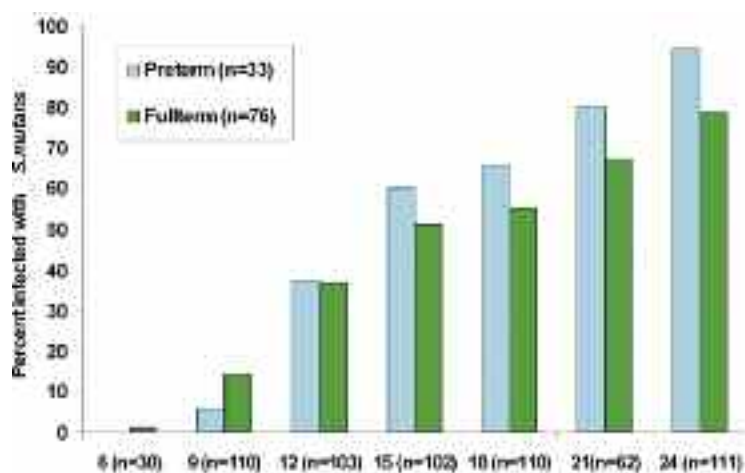
small blocks of paraffin to dislodge plaque so that it is dispersed into the saliva (Fig. 1).

All culture-based tests used in caries risk assessment have several limitations:

- Live, viable bacteria are necessary.
- An incubator is required to provide the optimum temperature for growth (37 degrees Celsius).
- It takes a substantial time (typically 48 hours) to obtain a result.
- The shelf life of the kit is limited because of temperature-related decline in some components of the growth medium (particularly the antibiotic bacitracin).
- MSB has been shown to under estimate the actual levels of S. mutans in samples.
- The growth media are semi-selective and there is little or no discrimination between S. mutans and S. sobrinus. A recent comprehensive assessment of the most suitable culture media for the enumeration of S. mutans from clinical samples determined the specificity and sensitivity of five so-called selective media.<sup>82</sup> Tryptone-yeast-cysteine-sucrose-bacitracin (TYCSB) was found to be the medium of choice for the isolation of S. mutans from saliva samples, giving the best delineation between S. mutans and both S. sobrinus and non-mutans streptococci (Fig. 2), however this medium is not available currently for chairside use. Confirmation of identity typically requires follow-up biochemical tests conducted in a panel using a set of fermentation and enzymatic tests, such as production of acid from N-acetylglucosamine, arbutin and melibiose, and the presence of alpha-galactosidase and alpha-glucosidase activities.<sup>83</sup> (Fig. 1, panels E and F).
- Atmospheric conditions for culture stipulate a low oxygen tension. For clinical samples, this often entails using expired air from the mouth, sodium bicarbonate pellets, or depleting oxygen using combustion or a catalyst. In contrast, in the laboratory setting, incubation of culture plates for mutans streptococci can be undertaken in an anaerobic environment (e.g. 95% nitrogen and either 5% carbon monoxide or 5% carbon dioxide).

**Sampling methods for culture-based tests**

An important variable in culture-based methods is the requirement for viable organisms, which replicate to form colonies. For precise measurement of colony forming units (CFU), standardized conditions of culture are required. This makes both clinical and field studies problematic in that storage and transport of growth media may influence viability and thus colony counts.



**Figure 5: Time course of colonization with *Streptococcus mutans* after tooth eruption, using tryptone-yeast-cysteine-sucrose-bacitracin (TYCSB) agar to assess samples of saliva. Bacterial identification was verified using the Rapid ID32 biochemical test. Data show the frequency of colonized individuals in a cohort of 111 infants (35 pre-term and 76 full-term). By the age of 24 months, 84% of infants harboured *S. mutans*. Based on data from Ref. 28.**

As summarized in Table 2, there are several possible methods of sampling which can be used when estimating levels of MS in the oral cavity:

- Direct sampling of plaque (e.g. overlying carious lesions) using an absorbent cotton tip or sponge
- Sampling of stimulated whole saliva using a plastic stick or wooden spatula which is then withdrawn through the lips, as in the “strip mutans” method
- Collection of stimulated whole saliva in a cup, the contents of which are then poured over a solid agar growth medium or used to inoculate a liquid culture
- Sampling of unstimulated saliva using an absorbent cotton tip or sponge (e.g. in neonates)
- Sampling of both unstimulated saliva and plaque using an absorbent tip which is gently rubbed over the dorsum of the tongue, alveolar ridges, and labial and lingual surfaces of all erupted teeth (e.g. in young children)
- Sampling from the dorsum of the tongue using a loop which is drawn across the surface, based on work which shows that levels of MS in whole unstimulated saliva correlate significantly with levels in debris collected with a loop from the dorsum of the tongue.<sup>84</sup>
- Sampling from the dorsum of the tongue using a wooden tongue depressor, which is then impressed directly onto culture plates.
- An oral rinse using a fixed volume of transport medium, which is then used as the inoculum.

Sampling of the stimulated saliva is preferred because of its simplicity, however it must be stated once more that salivary levels are a surrogate measure for plaque levels of cariogenic bacterial species. While organisms from plaque will be dispersed into the saliva by chewing, deeper portions of the

plaque biomass, particularly the less aerobic environments in fissures and interproximal spaces, will contribute relatively less to salivary levels by simple mechanical dispersion during chewing. It is for this reason that salivary counts and levels in plaque do not correlate absolutely.

There is also the effect of surface area, in that an increase in the available hard surfaces will tend to result in elevated counts simply because hard surfaces are the preferred ecological niche for mutans streptococci. This is amply demonstrated by reports of dramatic increases in salivary levels of mutans streptococci following the placement of orthodontic brackets.<sup>85</sup> Further evidence is seen in a study of caries risk factors in elderly patients, which reported the salivary levels of MS as a function of the number of teeth.<sup>86</sup> When the raw data for salivary levels were adjusted for the number of teeth in the mouth, levels of MS (reported as CFUs/ml saliva per tooth) were significantly associated with coronal surface caries. It was suggested that reporting salivary bacteriological data as a function of tooth number and per mL of saliva could improve the reliability of bacteriological data in that it would account at least in part for surface area dispersion effects involved in salivary sampling.

### Antigen-specific assays

These tests utilize highly specific monoclonal antibodies, giving absolute specificity for the bacteria of choice, e.g. *S. mutans* or *S. sobrinus*. These antibodies can be used in number of diagnostic methods, including immunofluorescence, flow cytometry, latex agglutination, immunoblots and solid phase immunoassays. The former methods are laboratory-based, while immunoassays have been brought to clinical application in dental practice.

The GC Saliva-Check SM™ uses a combination of three highly specific anti-*S. mutans* monoclonal antibodies (SWLA-1,



2, and 3),<sup>87,88</sup> to increase binding and reduce the detection limit to 100,000 bacteria per mL of saliva, which is the recognized level for increased caries risk (Fig. 3). The tests are undertaken at chairside within a short time frame, and no special apparatus or techniques are required. Unlike culture-based tests, viable bacteria are not needed. Samples of saliva are collected and reacted with buffers to establish a constant pH (and thus charge for proteins), and detergents, for proper dispersal of the sample. The sample is then placed on a strip of nitrocellulose or other suitable material which is impregnated with monoclonal antibodies, which trap *S. mutans* bacteria, triggering a detection reaction. Because this is complete within 5 minutes, the results can be discussed with the patient at the same appointment.<sup>9</sup> Control reactants ensure that the detection chemistry is working properly, allowing the clinician to interpret a negative result with confidence (Fig. 4).

Such tests are particularly useful for tracking transfer of MS from mothers to infants, as part of assessing strategies designed to reduce the risk of early childhood caries.<sup>89</sup> Using such tests provides data which agree with conventional cultural tests, but within the same appointment rather than after several days.<sup>9</sup> Typical results with immunoassays reveal that one third of an adult population would have levels above 100,000 per mL, and that a small proportion of the population would have more than one million per mL. In a large study conducted

by Shi et al. in which nearly 2,000 human saliva samples were examined using the three species-specific monoclonal antibodies to detect and quantify *S. mutans* levels in human saliva, values ranged from less than 10,000 to a high of 36 million cells/mL. Over 15% of the saliva samples examined had counts over 500,000 cells/mL.<sup>88</sup>

Contemporary laboratory studies for analyzing cariogenic plaque typically employ molecular methods with specific primers for 16S rRNA genes and amplification in the polymerase chain reaction (PCR), for example, competitive PCR, nested PCR, and real time PCR, with the latter giving the most rapid results.<sup>90-94</sup> While giving accurate bacterial counts down to low detection limits of 100 organisms or less, because these methods require DNA extraction and complex thermo-cycling equipment, they cannot be used in the dental office setting.

**Novel strategies for manipulating cariogenic biofilms**

The final section of this paper focuses on novel means for interfering with changes in biofilms that increase their cariogenicity, highlighting recent developments in dental research which may hold potential for application in clinical practice for high risk patients, where combinations of various agents would be used with appropriate lifestyle changes (Table 3). The underlying concept is that, from the caries perspective, dental plaque biofilms range from benign (thin, immature) through to more harmful (thick, fermenting). A range of commercial products may assist in determining surrogate endpoints for agents which could influence biofilms (Table 2). With regard to infants, caries prevention should target specific behaviours which will influence the biofilm (Table 4), taking into account the ease with which cariogenic biofilms develop on the teeth in the early years of life (Fig. 5).

One important implication of the belief that caries is a multi-pathogen biofilm disease is that “single pathogen” approaches such as vaccines to *S. mutans* or its components (such as glucosyl transferase), and the use of genetically modified *S. mutans* (with deletions of glucosyltransferase enzymes (B/C/D), the phosphoenolpyruvate-dependent phosphotransferase system (PTS), or of lactate dehydrogenase) would no longer be appropriate.

**A. Xylitol**

Xylitol is a naturally occurring polyol which is taken up by MS but is not fermentable. Numerous studies have documented the non-cariogenicity of xylitol,<sup>95</sup> however more recently attention has been directed to the effects of prolonged consumption of xylitol on MS. Chewing a xylitol gum three times daily for a minimum of five minutes each time for three months may give a 10 fold reduction in salivary levels of MS,<sup>96,97</sup> a strategy which would be useful in high caries risk patients, orthodontic patients and mothers of infants, as an adjunct to other suppressive approaches such as chlorhexidine therapy.

**Table 3: Caries prevention agents which influence biofilm behaviour**

**Traditional agents**

- Mechanical oral hygiene (biofilm disruption)
- Surfactants (in dentifrices and mouthrinses)
- High concentration and low pH topical fluorides
- Metallic ions (Cu, Fe, Zn, Sn, Ag)
- Oxygen radicals (ozone, hydrogen peroxide)
- Bisguanides - chlorhexidine
- Naturally occurring phenolics (including essential oils)
- Synthetic phenolics (Triclosan)

**Novel agents**

- Xylitol
- Casein Phosphopeptides
- Other natural plant-derived products (ingestible antimicrobials)
- Inhibitory bacterial species (probiotics)
- Alkalinizing approaches (arginine, urea)
- Synthetic peptides with antibacterial properties
- Agents which modulate biofilm behaviour (quorum sensing)
- Photosensitization

Habitual consumption of xylitol in the diet appears to select for MS with impaired adhesion properties, i.e., they bind poorly to teeth and shed easily from plaque to saliva. One important advantage of this approach is that it is suitable for maintaining long-term suppression of pathogens without concerns of safety with prolonged use. Use of high xylitol containing gums such as Xylimax™ (0.6 grams per pellet of gum) is a simple measure to incorporate into a daily routine, and brings the important advantages of increased salivary clearance, buffering and remineralization. This needs to be balanced against the issue of adaptation to xylitol in the diet.

### B. Natural peptides

Casein phosphopeptides (CPP) are naturally occurring molecules from bovine milk which are able to bind calcium and phosphate ions and stabilize amorphous calcium phosphate (ACP). Under acidic conditions, CPP are able to release calcium and phosphate ions and thereby maintain a state of supersaturation with respect to tooth enamel, reducing demineralization and enhancing remineralization.<sup>98</sup> The delivery of CPP or complexes of CPP and ACP (Recaldent™) to the plaque fluid can be achieved by a range of vehicles, including chewing gums, dentifrices and topical gels, such as GC Tooth Mousse and Tooth Mousse Plus (designated MI Paste and MI Paste Plus in some countries). CPP bind strongly to dental plaque, and are able to slow or prevent the diffusion of calcium ions from enamel during episodes of acid challenge, and serve as a source of calcium for subsequent remineralization<sup>99-101</sup>. CPP may also affect adhesion of MS and modulate fermentation by dental plaque bacteria.<sup>101-102</sup> An important aspect of CPP is their long half-life in saliva, which is due to their unique amino acid composition and to the release of phosphate ions which may inhibit proteolytic breakdown of these peptides. Their metabolism in plaque (with a half life of 2.8 hours) also results in a pH elevating effect because of their substantial arginine content.

### C. Synthetic peptides

Peptides with antimicrobial properties can also be synthesized, with inherent antimicrobial properties or as delivery systems for established biocides or toxins. Issues with these synthetic agents include: low binding affinity to plaque, poor penetration, limited stability in the biofilm because of proteolysis, low specific activity, and high cost. For these reasons, antibacterial peptides (bacteriocins) from oral bacteria would seem a more fruitful avenue to explore.

### D. Natural plant products

A number of plant-derived extracts which has been shown to possess significant anti-cariogenic properties. Propolis, essential

**Table 4: Risk factors for early colonization with cariogenic bacteria**

#### Infant factors

- Premature birth
- Enamel hypoplasia
- Sweetened fluids taken at bedtime
- Drinking only from bottles
- On-demand formula feeding
- Delayed start for solid foods
- Sucrose exposure more than 3X per day
- Sharing food and utensils with adults
- Regularly kissed on the lips
- Irregular toothbrushing by carers

#### Maternal factors

- High plaque levels
- Sucrose exposure more than 3X per day
- High salivary levels of *S. mutans*

Based on Ref. 28.

oils and flavonoids have been of interest for many years, with recent attention being directed towards cranberry extracts and the combination of the agents apigenin and farnesol, which block expression and secretion of the glucosyl transferase enzyme from MS, preventing their synthesis of insoluble glucans. Combinations of these agents with fluoride may allow a change in biofilm behaviour without necessarily reducing the levels of MS or other bacteria.<sup>103,104</sup>

Cacao bean husk extract (CBH) is one such material which under laboratory conditions can reduce the growth rate of MS and other oral streptococci, reduce acid production by MS, inhibit the synthesis of insoluble glucans by the glucosyltransferases from *S. mutans* and *S. sobrinus*, and thereby impair sucrose-dependent cell adherence. In the rat animal model of dental caries, administration of CBH in drinking water at concentrations exceeding 1.0 mg/mL has been shown to cause significant reductions in both plaque accumulation and in dental caries.<sup>105</sup>

Chewing sticks from the tree Juglandaceae regia have been used in the Indian subcontinent to maintain oral hygiene. Laboratory and clinical studies of the aqueous extracts from these sticks show a variety of anti-cariogenic properties, including reduced growth and adherence of *S. mutans* for up to 3 hours; reduced acid production by *S. mutans* for 90

minutes; reduced glucan-induced aggregation of *S. mutans*, and direct bactericidal effects on *S. mutans*.<sup>106</sup> An assessment of the aqueous extract from *Terminalia chebula* by the same authors yielded similar properties. The extract strongly inhibited the growth, acid production, sucrose induced adherence and glucan induced aggregation of *S. mutans*.<sup>107</sup>

### E. Alkalinization strategies

Increasing alkali production in dental plaque is a simple means of applying ecological pressure to the biofilm. *S. sanguis* and *S. gordonii* use arginine deiminase to elevate pH through metabolism of amino acids, while urease breaks down urea releasing ammonia which again elevates pH. Supplying substrate for these two enzyme pathways is the basis for poly-arginine (arginine bicarbonate/calcium carbonate, CaviStat™) and V-6™ chewing gum, respectively. While the urease gene from *S. salivarius* has been inserted into a genetically modified strain of *S. mutans* to make it ureolytic,<sup>108,109</sup> acceptance of genetically modified bacteria by the community as a dental intervention would likely pose major problems.

### F. Inhibitory organisms and probiotic approaches

A number of novel strategies exploit competition between bacterial species. This can be direct (through secretion of bacteriocins, toxins, enzymes or waste products) or indirect (through environmental changes in pH). Organisms of interest have included clinical isolates of *S. sanguis*, *S. salivarius* (strain K12) and *S. oligofermentans*. The probiotic approach makes sound biological sense, provided the organisms can be delivered often enough to establish their presence over their commensal counterparts.

In fact, there is increasing evidence of an important interaction between oral bacteria within the sanguinis (sanguis) group and MS.<sup>110</sup> These bacteria have the advantage of being naturally derived, normal commensals, and thus come under the “generally recognized as safe” (GRAS) regulatory classification. A recent study demonstrated that there may be competition and antagonism between *S. sanguinis* and MS.<sup>111</sup> Children who did not harbour detectable levels of MS had higher levels of *S. sanguinis* in their saliva than children colonized with MS. If *S. sanguinis* (or other bacterial species) were to be used as a probiotic therapy in a topical product, issues of shelf life (for a product containing live bacteria) would provide an important technical challenge to overcome.

Screening libraries and culture collections of food-derived bacteria for inhibitory effects on dental caries has also become a major area of investigation in recent years, aiming to find agents which are GRAS and thus free of safety

concerns for repeated ingestion. Several species have been identified which can cause lectin-glycoprotein adhesion to *S. mutans*, raising the possibility of reducing levels of these in saliva and the superficial layers of dental plaque after using rinses or dentifrices to aggregate *S. mutans* and the probiotic bacteria into clumps which are then ingested or expectorated.<sup>112</sup> These adhesive interactions should be effective even if the probiotic bacteria have been inactivated (for long term storage) by pasteurization, as the lectins would be heat resistant. The same approach could also be taken with delivery using sugar-free confectioneries. Current species of interest include certain isolates of *Lactobacillus paracasei* and *Lactobacillus rhamnosus*.

### G. Altering biofilm communication pathways

Biofilm properties may also be manipulated by affecting the pathways of biofilm manipulation, or of cell to cell signaling within the biofilm. Blocking this “quorum sensing” would reduce the ability of the biofilm to tolerate stresses such as reductions in nutrients or assault by external chemical agents (such as biocides). Slowing the biofilm accumulation rate may be possible using agents such as furanone which affect quorum sensing.<sup>113</sup>

### H. Targeted therapies

“Magic bullet” and “smart bomb” therapies are of ongoing interest. Antibodies to particular bacterial species in the biofilm could be conjugated to a toxin or biocide, however the challenges remain of gaining penetration and resisting degradation by proteases within the plaque biofilm, which may prove to be insurmountable. Using endogenous photosensitization of protoporphyrins in biofilm bacteria<sup>114-117</sup> with light as the vector may overcome the penetration problem, and remains an important area for further investigation.

### Conclusions

There are now multiple lines of evidence which indicate that dental caries is a multi-pathogen disease and that MS may participate but are not critically essential for disease to occur. Taking a “whole of biofilm” ecology approach to dental caries introduces a number of new strategies for controlling caries risk. Clinical diagnostic kits can be valuable in patient management provided their interpretation as surrogate measures of the disease process is borne in mind. Modern technology for plaque fermentation testing and for chairside immunoassays fits well within contemporary caries management strategies such as STEM (System for Total Environmental Management)<sup>6</sup> and CAMBRA (Caries management by risk assessment).<sup>118-119</sup> Dental hygienists,

dental therapists and dental chairside assistants can assist the dentist or dental specialist in the caries management process through their delegated work using chairside tests for patient workup and monitoring.

## Disclosure

LJ Walsh was responsible for developing the GC Plaque-Check+pH test for dental plaque fermentation, and has a commercial interest in this diagnostic kit. Both authors have been involved in clinical trials using culture-based and immunoassay tests from several manufacturers, but neither have any commercial interest in these products.

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