Community Dent Oral Epidemiol 2005; 33: 248–55

COMMUNITY DENTISTRY AND ORAL EPIDEMIOLOGY

The complex oral microflora of high-risk individuals and groups and its role in the caries process

David Beighton

Department of Microbiology, Dental Institute, King's College London, London, UK

Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol 2005; 33: 248–55. © Blackwell Munksgaard, 2005

Abstract – The involvement of the oral biofilm in the caries process requires re-evaluation. The essential role of mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) in the caries process is not proven. Acid production by dental plaque is not dependent upon the presence of mutans streptococci; caries occurs in the absence of these species and their presence does not necessarily indicate caries activity. Other oral bacteria, non-mutans streptococci, *Actinomyces* spp. and *Bifidobacterium* spp., are acidogenic and aciduric. They outnumber mutans streptococci in dental plaque, and there are data which support a role for these bacteria in the initiation and progression of caries. Molecular studies demonstrate the great diversity and complexity of the flora associated with caries. Many taxa identified have not been cultured and the role of these taxa is not known. We have, in mutans streptococci, good markers of disease but not necessarily the aetiological agents of the disease. Considerably more research is required to investigate the transition of tooth surfaces from being intact and sound to the white spot lesion stage. A combination of conventional and molecular approaches are required to elucidate the involvement of an individual taxon and of microbial populations with particular traits in the caries process.

Key words: acidogenicity; aciduricity; *Actinomyces*; dental caries; dental plaque; mutans streptococci

David Beighton, Department of Microbiology, The Henry Wellcome Laboratories for Microbiology and Salivary Research, Dental Institute, Kings College London, Floor 17, Guys Tower, London Bridge, SE1 9RT, UK
Tel: +44-(0)2071887465
Fax: +44-(0)-2071887466
e-mail: david.beighton@kcl.ac.uk

Submitted 14 September 2003; accepted 14 February 2005

Sophisticated genetic studies involving the sequencing of amplified 16S rRNA genes extracted directly from plaque and saliva samples have indicated the oral microflora to belong to over 500 different taxa. A large proportion of these taxa probably have not been cultured using conventional microbiological plating techniques (1). The majority of these bacteria accumulate on the dentition irrespective of the sucrose content of the diet, and a majority of supragingival plaque bacteria has the ability to produce acids from the sugars commonly found in human diets. However, there is a large and distinguished body of research which has attempted to identify mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) as the major bacterial agents of tooth decay (2, 3). This may not be the case. In this concise review, the evidence that other bacteria, or perhaps more correctly other microbial populations, are involved in the caries process, and that the mutans streptococci are primarily markers of disease, will be presented. The need for future studies to explore the microflora associated with caries on enamel and dentine surfaces in people of different ages cannot be over-emphasized nor should our ability to undertake such studies be under-estimated.

The specific plaque hypothesis

Dental caries is the consequence of the interaction between the oral microflora, the diet, the dentition and the oral environment (4). Bacteria are crucial to the initiation and progression of carious lesions. Without bacteria there are no lesions. For many

years until the late 1940s, lactobacilli were regarded as the main microbiological agents of tooth decay. However, the longitudinal study by Hemmens et al. (5) clearly demonstrated that lactobacilli colonized lesions and that they were not prevalent in plaque during the period of lesion formation. A careful and well-designed study had clearly shown that these bacteria could not be regarded as the major aetiological agents of tooth decay.

Studies using germ-free rats orally inoculated with single strains of bacteria revealed that not all bacteria were capable of forming carious lesions in rats fed diets containing high levels of sucrose (6). These and similar studies demonstrated that a range of bacteria, including *Streptococcus salivarius* and *Enterococcus* spp. which are not normally isolated in large numbers from dental plaque, would cause caries in rats. It was clear from these studies that a hierarchy existed, with *S. mutans*, isolated from cavitated carious lesions by Clarke (7), as the most cariogenic of all the species tested in the rat models. This pattern was observed when strains were inoculated into germ-free SPF or conventional rats fed a diet containing a high level of sucrose. The mutans streptococci were the most cariogenic and other commensal bacteria, streptococci and *actinomyces*, were less cariogenic. *Actinomyces viscosus* was particularly associated with root caries in gingivectomized rats (8) and some strains of *Streptococcus oralis* and '*Streptococcus milleri*' exhibited levels of caries induction in rats that approached those of the mutans streptococci (9). There was no explanation for these findings. Of course it must be remembered that to be cariogenic in a germ-free rat, the implanted organism must, on its own, colonize teeth, while in the human mouth such organisms may colonize as a consequence of their interactions with other dental plaque bacteria.

These studies enabled the formulation of hypotheses regarding the role of mutans streptococci in the human caries process. Numerous studies have demonstrated an association between the number and concentration of mutans streptococci in plaque and/or saliva and the dental caries status of populations, and to a lesser extent to the caries status of an individual (10, 11). Caries occurs in the absence of mutans streptococci (12) and individuals with high levels of mutans streptococci do not necessarily have to have caries (13).

Caries-associated characteristics of mutans streptococci

Laboratory investigations into the phenotypic characteristics of mutans streptococci which might explain their apparent involvement in the caries process revealed a number of characteristics which were related to their interactions with sucrose and to their ability to produce large amounts of acid (acidogenicity) rapidly, to tolerate exposure to low-pH environments (aciduricity) and to produce acid in an already acidic environment. In comparison with other bacteria tested *in vitro*, the mutans streptococci are generally able to produce acid most rapidly, to produce the largest amount of acid when exposed to a low-pH environment (pH ≤5.5), and to survive exposure to acidic conditions (14, 15). In such tests, *S. sobrinus* is more acidogenic and more aciduric than *S. mutans*, yet this species can be subcultured from only a minority of subjects, and is usually accompanied and outnumbered by *S. mutans*. Although polymerase chain reaction (PCR) detection methods indicate that *S. sobrinus* may be more prevalent than indicated by cultural studies, it is rarely present at the same level in plaque as *S. mutans* (16). The reason for the inability of *S. sobrinus* to proliferate appears to be due to its inability to catabolize transported *N*-acetylglucosamine, an energy-requiring process, which depletes intracellular levels of phosphoenolpyruvate to the detriment of the organism (17). Only when external sources of fermentable carbohydrates are high, or the oral environment is very acidic as in bulimic subjects (18), does this inhibitory effect become insignificant and *S. sobrinus* proliferates.

The mechanisms underlying the aciduricity of *S. mutans* have been investigated using proteomic and genetic methods (19, 20) but to date no precise mechanism has been identified, although many genes or proteins were found to be apparently involved, especially in survival at low pH. It is unlikely that the expression of any single gene is solely responsible for the aciduricity of mutans streptococci.

Mutans streptococci also possess a number of glucosyltransferases, which have the ability to form a range of distinct polymers from sucrose. Uniquely, mutans streptococci produce a highly branched, water-insoluble glucan, mutan, which may facilitate its establishment in dental plaque. These polymers are necessary for the adherence of *S. mutans* to the enamel, and in rat studies mutants

with these genes inactivated and unable to produce these polymers are less able to colonize the dentition of rats. Consequently, they are less able to initiate carious lesions (21, 22).

It is clear then that the ability to produce acid and to tolerate a low-pH environment is essential for an organism to initiate and progress tooth decay. However, that the ability to produce polymers from sucrose is an essential virulence characteristic for other bacteria which might be involved in the initiation of dental caries it is not at all apparent.

Is there evidence that bacteria, other than mutans streptococci, with such acidogenic and aciduric properties exist? It is difficult to obtain pertinent information on these points, as so much emphasis has been placed on enumerating mutans streptococci in dental plaque and analysing the physiological properties of these organisms. However, a study performed on elderly Chinese subjects is illuminating (23). The investigators related the sucrose-induced pH response of the oral biofilm (dental plaque) on sound exposed root surfaces to the microbial composition of the overlying plaque. The prevalence of a range of cultivable taxa, including *S. mutans* and *S. sobrinus*, was determined, and the pH of the plaque was measured before and after the application of a sucrose rinse in 17 elderly Chinese with poor oral hygiene. The authors found no difference in plaque pH response on sound and carious root surfaces and the pH response to sucrose was the same, regardless of the presence or absence of mutans streptococci (Fig. 1).

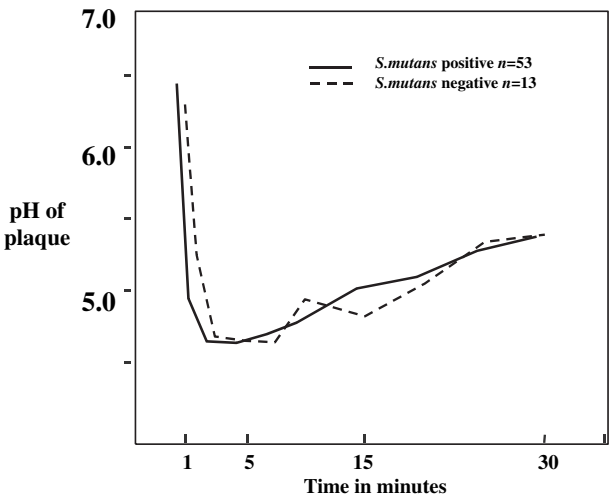

Fig. 1. Comparison of the acid response (Stephan curve) of plaque positive or negative, by culture, for mutans streptococci (figure provided by Professor Anne Scheie).

In plaque harbouring mutans streptococci, the mean Stephan curve was not statistically different from that produced by plaque exposed to sucrose which did not harbour mutans streptococci. It is apparent from this study that the rate of acid production from dental plaque is not determined by the presence of mutans streptococci, nor is the final pH recorded following the sucrose rinse determined by the presence of mutans streptococci in the oral biofilm. These observations are in contrast to those derived from *in vitro* studies using single strains of planktonic organisms, and in this perhaps lies some of the limitations of such studies. The bacteria on the teeth grow in a biofilm and they grow as part of a complex interacting microbial community. None of these features are included in many of the *in vitro* studies which found the acidogenicity of mutans streptococci to be significantly greater than that of other bacteria isolated from dental plaque. On the basis of the results from the Chinese subjects, the investigators concluded that the caries process was not mediated by the presence of mutans streptococci, and that other bacteria present in the oral biofilm were responsible for caries initiation and progression in these subjects.

Acidogenicity and aciduricity of bacteria recovered from dental plaque

Acidogenicity

Studies to compare the acidogenicity and aciduricity of dental plaque bacteria have investigated bacteria isolated on conventional culture media which are often subject to repeated subculturing in the laboratory. Such bacteria may have undergone adaptation to their new environment, laboratory culture media, and may no longer exhibit the characteristics they displayed *in vivo*. To overcome these potential difficulties, studies have been devised to examine the properties of bacteria isolated from dental plaque and subjected to minimal subculturing.

A conventional plate assay to enumerate and quantify the acidogenic populations in dental plaque was undertaken (24). The authors determined that the acidogenic composition of dental plaque reflected the dietary habits and caries status of an individual, and that a high acidogenic ratio was associated with active lesions or recent restorations. Individuals who were caries-free had a lower ratio, although the total number of bacteria

were similar. Of interest is that the acidogenic population was increased by frequent exposure to sugar. Furthermore, streptococci, *Actinomyces* and *S. mutans* were isolated amongst the acidogenic flora from all samples, but no consistent relationship was found between either their isolation frequency or proportions and caries status. These data suggest that the acidogenic population in plaque was varied and that bacteria other than mutans streptococci increased in plaque in response to increased frequency of consumption of sucrose.

van Houte et al. (25–27) investigated extensively the acidogenicity of bacteria isolated from a variety of sound and carious tooth sites. They isolated bacteria on nonselective media and then determined the acidogenicity of numerous strains from different sites. The acidogenicity of the isolates was determined by measuring the final pH of 7-day-old cultures initially containing 1% glucose. In both coronal caries and root-surface caries, they identified significant populations of streptococci which produced large amounts of acid (pH <4.2 in broth) and were termed 'low-pH non-mutans streptococci' (25–27). They suggested that these organisms, presumably including *S. gordonii*, *S. oralis*, *S. mitis* and *S. anginosus*, outnumber the mutans streptococci in virtually all plaque samples examined, and so they suggested that these bacteria may play a significant role in the caries process. They also found – and this was the most significant aspect of their studies – that the non-mutans streptococci were heterogeneous with respect to acidogenicity. It had been presumed prior to their investigations that the terminal pH that individual species attained in culture was particular to that species and that there was little variation in the pH values attained. They demonstrated that this heterogeneity had a clinical significance, being associated with the disease status of the site sampled. There was a selection for more acidogenic strains of non-mutans streptococci within carious lesions compared with those isolated from plaque on sound tooth surfaces. These data are summarized in Table 1. It can be seen that the lactobacilli and mutans streptococci all produced a terminal pH in broth that was <4.2. The non-mutans streptococci present a different picture, where 78 of 100 isolates from root lesions produced a final pH of <4.2. Only 16 of 100 isolates from sound root surfaces produced this low pH in culture. The proportion of *Bifidobacterium* was also greater from the lesion samples; this issue will be discussed later.

In more recent publications (28, 29), it was concluded that the trend towards plaque containing increased numbers of bacteria with greater acidogenic and aciduric properties was driven by increased carbohydrate consumption, and is concurrently accompanied by an increase in the proportion of polysaccharide-storing bacteria. The authors suggest a succession in the microflora resulting in caries caused by increased fermentable carbohydrate fermentation, in which many different bacterial taxa respond to the changing environment and increased numbers exhibit a greater ability to produce and withstand exposure to acid. They found that the emergence of mutans streptococci in plaque was often preceded by an increase in the number of other types of acidogenic bacteria, which included not only the non-mutans streptococci but also other members of the plaque flora which were not necessarily streptococci. Understanding that caries is a consequence of many interacting factors it was recognized that the initiation of caries, in the absence or presence of mutans streptococci, could be explained by the dynamic and positive relationship among the factors of carbohydrate consumption, plaque flora composition, plaque acidogenic potential and caries activity.

The studies by van Houte et al. (25–27) underline and reveal the intricate relationships between diet,

Table 1. Comparison of the percentage distribution of lactobacilli, mutans streptococci, bifidibacterium and non-mutans streptococci, isolated from root caries lesions ($n = 84$) and sound tooth surfaces ($n = 223$) on the basis of their terminal pH values in 1% glucose broth [from van Houte et al. (25)]

	Terminal pH in 1% glucose broth					
	Advanced root caries lesions			Sound root surfaces		
Organism	<4.2	4.2–4.4	>4.4	<4.2	4.2–4.4	>4.4
Lactobacillus	100	0	0	0	0	0
Mutans streptococci	100	0	0	100	0	0
Bifidobacterium	68	11	21	0	0	0
Non-mutans streptococci	78	18	4	16	51	33

the microflora, and caries. They clearly demonstrated the heterogeneity of oral bacteria and the capacity of these to respond to the impact of dietary carbohydrates, and the consequent increase in acid production, by in turn exhibiting increased acidogenicity.

Aciduricity

The genotypic heterogeneity of *S. oralis* with respect to aciduricity was investigated using repetitive extragenic palindromic PCR (30). In this study, individual plaque samples were cultured in media adjusted to pH 5.2 and the predominant taxa isolated at this pH determined. *S. oralis* was the predominant aciduric bacterium isolated from noncarious tooth sites. The aciduric *S. oralis* isolates represented 1.9% (±0.7%) of the cultivable bacteria in the plaque samples and, on average, the *S. oralis* strains isolated in the media at pH 5.2 represented approximately 50% of the bacterial count. The *S. oralis* isolates recovered from the Mitis Salivarius Agar (MSA) plates formed 11.2% (±3.6%) of the total count from the nonselective media and these isolates were compared with those from MSA. It was found that unrelated subjects harboured unique genotypes of *S. oralis* and each person harboured numerous genotypes. Genotypic comparison of the aciduric populations isolated at pH 5.2 with those isolated from MSA indicated that the aciduric populations were genotypically distinct in the majority of subjects ($\chi^2 = 13.09$; $P = 0.0031$). While no association between the presence of these populations and caries was investigated, these studies provided further evidence of the physiological and genotypic heterogeneity of non-mutans streptococci. However, the authors concluded that the presence of distinct aciduric populations of *S. oralis* implies that the role of these and other non-mutans streptococci in the caries process requires re-evaluation. These observations support and complement the observations by van Houte et al. (25–27).

The same cultural methods were used to study the aciduric flora associated with root caries (31). In this study, plaque was taken from sound exposed root surfaces in subjects with no root caries activity, from sound root surfaces in subjects with root caries, and from root carious lesions. All samples were collected from different subjects and these were cultured on conventional media as well as in media adjusted to pH 4.8 or 5.2. It was found that the proportion of the flora that was isolated at pH 4.8 was significantly less from the sound surfaces in subjects with no root caries (0.008%) compared with the levels in the other groups, which were not significantly different (Table 2). At pH 5.2, the recovery from the lesions was significantly greater than that from the other two sites. From none of these samples were mutans streptococci recovered amongst the predominant aciduric bacteria. This study shows that the most diseased sites and those most at risk harboured the largest populations of aciduric bacteria while the sites in subjects at least risk harboured the smallest populations of aciduric bacteria.

Table 2. Aciduric flora isolated in media at pH 4.8 and 5.2 as a percentage of the count in media at pH 7.0

	pH 4.8 as per cent of pH 7.0	pH 5.2 as per cent of pH 7.0
Root caries ($n = 14$)		
Mean	21.6	12.6
SE	8.3	4.1
Median	9.6	5.3*
Sound root surfaces in subjects with root caries ($n = 15$)		
Mean	15.9	16.1
SE	8.9	8.1
Median	1.2	0.8
Sound root surfaces in subjects without root caries ($n = 10$)		
Mean	1.4	2.4
SE	1.4	1.6
Median	0.008*	0.25

*$P > 0.05$ from other values in the column.

Consideration of the flora recovered from the media adjusted to different pH values is shown in Table 3. The flora was different between the three different sites, and different within each site according to the pH of the isolation medium. Lactobacilli represented 56% of the isolates recovered from lesions using the media at pH 4.8 and were not recovered among the aciduric isolates from either of the other sites. The recovery of *Actinomyces naeslundii* from lesions was pH-dependent comprising 9% of isolates at pH 4.8, 12.8% at pH 5.2, and 37.4% at pH 7.0, while the recovery of *Actinomyces israelii* and *Actinomyces gerencseriae* remained relatively constant from the lesions, irrespective of the pH of the medium.

The predominant aciduric flora from the two groups of sound exposed root surfaces was distinct and differed from the predominant microflora from the lesions. In the plaque samples from root surfaces in subjects with root caries the predominant aciduric species was *A. gerencseriae*. This species was isolated from a majority of subjects and comprised 63.3% and 61.9% of isolates at pH 4.8

Table 3. Identity of bacteria recovered in media at different pH values, expressed as a proportion of isolates, from root lesions and sound root surfaces

	Site		
	Root caries lesions	Caries-free in subjects with root caries	Caries-free in subjects with no root caries
Media at pH 4.8			
Lactobacilli	56.0	0	0
A. naeslundii	9.0	2.0	0
A. gerencseriae	3.7	61.9	17.6
A. israelii	18.7	3.4	0
S. oralis	3.0	0	0
S. anginosus	0	0.7	56.9
S. salivarius	0	25.2	0
No. of isolates identified	134	147	51
Media at pH 5.2			
Lactobacilli	31.7	0	0
A. naeslundii	13.8	4.3	0
A. gerencseriae	5.7	59.4	11.6
A. israelii	15.4	0	0
S. oralis	8.1	0	40.6
S. anginosus	0	0.7	23.2
S. salivarius	1.6	29.0	8.7
No. of isolates identified	123	138	69
Media at pH 7.0			
Lactobacilli	28.2	0	0
A. naeslundii	37.4	12.6	1.4
A. gerencseriae	3.1	2.7	2.8
A. israelii	13.0	0	0
S. oralis	5.3	17.1	33.3
S. anginosus	0	1.5	2.8
S. salivarius	0	23.4	21.1

and 5.2, respectively, but only 2.7% of isolates at pH 7.0. In contrast, this species was isolated from the root surface plaque sample from only one subject without root caries. *S. salivarius* formed approximately 25% of isolates from the root surface samples from subjects with root caries at pH 4.8 and 5.2, while from the subjects without root caries this was <10% at these pH values. *S. oralis* was not isolated from the samples taken from sound root surfaces in the subjects with root caries at pH 4.8 and 5.2, but formed 17% of the isolates at pH 7.0. In the samples from subjects without root caries *S. oralis* was not isolated at pH 4.8 but comprised 40.6% and 33.8% of isolates at pH 5.2 and 7.0. A similar trend was noted in the recovery of *S. parasanguis* from subjects without root caries. The other notable change in recovery was the reduced representation of *S. anginosus* amongst the isolates from the samples taken from subjects without root caries as the pH of the isolation medium increased.

These data demonstrate a number of things regarding the aciduric microflora isolated from root caries lesions and from exposed sound root surfaces. First, the microflora is different from all three sites; secondly, mutans streptococci are not amongst the predominant aciduric microflora; and thirdly, the flora isolated at more acidic pH values is different to that isolated at pH 7.0, the approximate pH of nonselective microbiological culture media. These data also suggest that certain *Actinomyces* spp., particularly *A. gerensceriae*, are involved in the initiation of root caries lesions as it was isolated as the predominant aciduric species on the root surfaces of subjects with root caries.

Together these data, derived using essentially conventional culture techniques supplemented with an assessment of genotypic and physiological characteristics, provide evidence that the microflora is heterogeneous. Individual species may be heterogeneous with respect to acidogenicity and aciduricity, features of mutans streptococci used to implicate these species in the caries process. While the associations between caries and the proportions of bacteria with acidogenic or aciduric traits were demonstrated, these studies provide only a basis for designing better investigations to enable to deeper understanding of the involvement of bacteria in the process of lesion formation. The changes in the flora from sound surface to white spot lesion through to cavitated lesion are not at all understood. The cross-sectional studies clearly suggest microbial succession between these stages, although a definitive description of these flora and their physiological characteristics need to be determined.

Molecular characterization of caries-associated dental plaque

To provide a more comprehensive understanding of the microflora associated with caries, modern genetic methods have been developed that do not require the conventional isolation of bacteria. However, such noncultural methods do have their limitations. Genotypic variation cannot be determined, nor can the physiological properties of taxa identified be characterized. In addition, there are issues surrounding the processes of DNA extraction and amplification of the 16S rRNA genes. Nonetheless, these approaches are potentially powerful instruments.

One approach, which although not yet reported to have been applied to a study of caries, is the use of denaturing gradient-gel electrophoresis (DGGE) to investigate the microbial composition of dental plaque. McBain et al. (32) have reported the use of DGGE to investigate the composition of dental plaque microcosms grown *in vitro*. The artificial plaque was sampled, the DNA extracted, and the 16S rDNA (rRNA gene) amplified using eubacterial-specific primers. This gave rise to a population of PCR amplicons that were separated by DGGE. Individual amplicons, corresponding to individual taxa, were visualized on the gels and these were excised and sequenced. The sequences were BLAST-searched and the species from which each was derived determined. Although few amplicons from the *in vitro* plaques were sequenced, five of 11 that were reported corresponded to either novel taxa or to uncultured members of known taxa. The importance of this study is that it provides a method with the potential for investigating the predominant microflora of dental plaque using culture-free methods. Although there are limitations with this method it provides a different approach to examining microbial populations in their entirety without the need for isolating individual colonies.

A similar but more powerful approach was reported by Becker et al. (33), who isolated DNA from dental plaque associated with sound and carious coronal sites in children. They amplified the rRNA genes present amongst the DNA and cloned the resultant amplicons into *Escherichia coli* which enabled each to be sequenced. In all, a total of 294 clones were sequenced and these belonged to 68 species or phylotypes of which 18 were uncultivated taxa and 10 were not previously identified. The most complex flora was associated with plaque from intact tooth surfaces. These data, and that from the DGGE study, demonstrate that even in these limited studies of 16S rRNA genes the complexity of the oral flora and the presence of numerous taxa which have yet to be investigated physiologically. But their presence cannot be ignored.

In a second part of their study, Becker et al. (33) used a reverse checkerboard assay to semi-quantify the numbers of 23 well-characterized species which might be associated with health or caries. A strong relationship was found between the numbers of *S. mutans* and caries but not between *S. sobrinus* and caries. *A. gerensceriae* was the most numerous organism in white spot lesions, and the authors suggest that this species maybe was associated with caries initiation. The closely-related species *A. naeslundii* followed a different pattern and was more associated with health than disease. Such observations follow those made previously concerning the involvement of both species with root caries (31). Overall this study demonstrated that, in addition to *A. gerensceriae* and *S. mutans*, *Bifidobacterium*, *Veillonella*, *S. salivarius*, *S. constellatus*, *S. parasanguis* and *Lactobacillus fermentum* were associated with caries. *A. gerensceriae* and other *Actinomyces* spp. appeared to be associated with caries initiation. A novel *Bifidobacterium* sp. may represent a major pathogen associated with deep caries lesions, thus complementing the acidogenic data described previously (25).

These studies only serve to underline the complexity of the flora associated with caries and with caries initiation. They demonstrate that the proposed relationship between the presence of mutans streptococci and the aetiology of dental caries may be facile and simplistic. No matter how useful the relationship between salivary or plaque levels of mutans streptococci and caries is in clinical practice, it must be regarded as an association and not a cause-and-effect relationship. Caries occurs from a complex interaction between dental plaque (whose physiological characteristics may be modified by carbohydrates in the diet), the diet, and oral hygiene practices. These interactions are difficult to unpick and understand, especially as our knowledge of the microflora associated with health, disease and the transition from health to disease is not known. It must also be recognized that the microflora associated with the transition state between health and disease may be different on different enamel surfaces (occlusal and approximal) and on exposed dentinal root surfaces.

Conclusion

The microbial aetiology of coronal and root caries needs to be better understood. An approach combining conventional culture methods, supplemented with the physiological characterization of the indigenous microflora, in combination with molecular methods is required. But without careful clinical characterization of the tooth sites sampled, such complex microbiological studies will be futile. Future research may provide better specific

predictive tests based on physiological determinants that identify individuals or individual tooth sites at risk of tooth decay.

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